JOURNAL OF THE NATIONAL CANCER INSTITUTE

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TRIBUTE TO DR. JEROME T. SYVERTON

by

Members of the National Advisory Cancer Council

The sudden death of Dr. Jerome T. Syverton in New York City on January 28, 1961, at the age of 53, was a grievous shock to the host of his admirers and a calamity for many academic and national organizations which he served with ability and with a devotion that went to the limit of endurance. Among these debtor organizations are the National Institutes of Health and its component parts, particularly the National Advisory Cancer Council and the Panel on Viruses and Cancer, of which he was a charter member.

For 4 years, from 1950 to 1954, Dr. Syverton was a member of the Virus and Rickettsial Study Section of the National Institutes of Health. He was a member of the Enterovirus Committee, and, most importantly, was Chairman of the Cell Culture Collection Committee. He served a four-year term as a member of the National Advisory Allergy and Infectious Diseases Council, with a special interest in the adenoviruses. At the time of his death, he was serving as a Special Consultant to the National Institutes of Health. In addition, it is to be recorded that he was a member of the recent Virologist Commission to Russia.

In all of these and other scientific and medical relationships, Dr. Syverton was an active participant through his own research, teaching, and wise guidance. His work on the interaction between viruses and cells was a signal contribution to research on both cancer and poliomyelitis. In his leadership, he brought to bear on problems of cell culture and the study of viruses his intelligence and skill, and the superb and powerful staff and facilities that he had built up at the University of Minnesota School of Medicine, where, since 1948, he had been Professor of Bacteriology and Immunology, and head of the Department. The influence of his conceptions and scientific contributions, and the uplifting example of his earnestness, honesty, integrity, and character were not confined to either his University or these Institutes of Health. He was a person and a scientist of national and international importance.

The members of the National Advisory Cancer Council, assembled at their meeting Bethesda, Maryland, on March 6, 1961, treasuring the recollection of their privileged association with Dr. Syverton, paid tribute to him as their leader and dear friend. With strong and comprehensive sentiment they voted to enter upon the records of the National Advisory Cancer Council this expression of their respect for the memory of Dr. Syverton.

Editorial History of the Journal of the National Cancer Institute

DORIS M. CHANEY[1] *and* MARIE K. MURPHY,[2]
Editorial Office of the Journal of the National Cancer Institute,[3] Bethesda, Maryland

SUMMARY

This 20-year history of the *Journal of the National Cancer Institute* from volume 1 through 24 includes a description of the methods of scientific review and editing. An analysis revealed a threefold increase in pages and a 50-fold increase in manuscripts from other research institutions. Trends in research fields of manuscripts published in volumes 1 to 5 and 16 to 24 are compared, and the new *NCI Monograph Series* is discussed.—J. Nat. Cancer Inst. 26: 785–799, 1961.

THE *Journal of the National Cancer Institute* is one of three journals in the United States devoted exclusively to reporting the results of cancer investigations—*Cancer* and *Cancer Research* are the other two. The first issue was published in 1940, only a few years after the creation of the National Cancer Institute in 1937 by an act of Congress (*1*). This act authorized publication of the results of cancer research but left the choice of an appropriate medium to Dr. Carl Voegtlin, first Chief of the National Cancer Institute (1938–43). He decided to initiate a new journal—the *Journal of the National Cancer Institute*—for reasons stated in the original justification (*2*).

> Scientists are accustomed to having results of their work presented through the medium of a periodical issued at regular intervals . . . such periodicals are better adapted for permanent preservation in libraries and in laboratories . . . rapid dissemination of knowledge will be an effective tool in the coordination of cancer research. . . .

In response to requests for various types of information about the Journal, a brief history of its development in the past 20 years and a report on other phases of its publication are presented.

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[1] Managing Editor, 1954–; editorial assistant 1949–54.
[2] Monograph Editor, 1959–; editorial assistant 1957–59.
[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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HISTORY

1940–1948

The first bimonthly issue was published in August, 1940, and printed by the U.S. Government Printing Office. Dr. Voegtlin assumed the task of scientific review, but often asked scientists on his staff for advice. His able assistant, Carolyn Whitlock, did the technical editing until she retired in December, 1948. The excellence of the papers in the first volume set a standard for the caliber of future manuscripts. Though most of the papers were written by the staff, authors from other institutions were represented, and the inclusion of the proceedings of the First National Cancer Conference on Gastric Cancer established a precedent in the Journal for publishing this type of material.

The shortage of staff during the war soon reduced the number of manuscripts available from this source and, since only 12 percent of the papers in the first 5 volumes were by outside authors, it became increasingly difficult to meet publication schedules (text-fig. 1).

The problem of obtaining manuscripts had become acute by the time Roscoe B. Spencer, second Chief of the National Cancer Institute (1943–47), established an Editorial Committee and was one of the main considerations of the first chairman, Jesse P. Greenstein (late Chief of the Laboratory of Biochemistry). After a 6-month term, he was succeeded by Howard B. Andervont, of the Laboratory of Biology, who served in this capacity until the first Scientific Editor was appointed in 1949. These two men often met publication deadlines by encouraging completion of manuscripts being prepared by the staff. Sometimes they even dashed off papers themselves.

When Leonard A. Scheele became Director of the National Cancer Institute (1947–48), it was decided that an Advisory Board of scientists from other institutions would contribute new ideas and stimulate the submittal of manuscripts from other areas. Members of this Board and the Editorial Committee were listed in the Journal in October, 1947. Some decisions made in meetings of these two groups are still valid today, for example, the acceptance of clinical research papers and review articles and the stipulation that each article contain a paragraph relating the text to cancer research. Through their efforts publication continued.

In May, 1948, John R. Heller was appointed Director of the National Cancer Institute when Dr. Scheele became Surgeon General of the Public Health Service. Dr. Heller named Harry Eagle as Editor, and Miss Whitlock as Managing Editor, and encouraged them to work closely with the Editorial Committee and Advisory Board.

1949–1960

The expansion of research in 1949 brought a request from Rolla E. Dyer, Director of the National Institutes of Health, for an Editorial Committee in each institute. The purpose of this action was the review

Carl Voegtlin
First Chief
National Cancer Institute
Founder of the *Journal of the National Cancer Institute*

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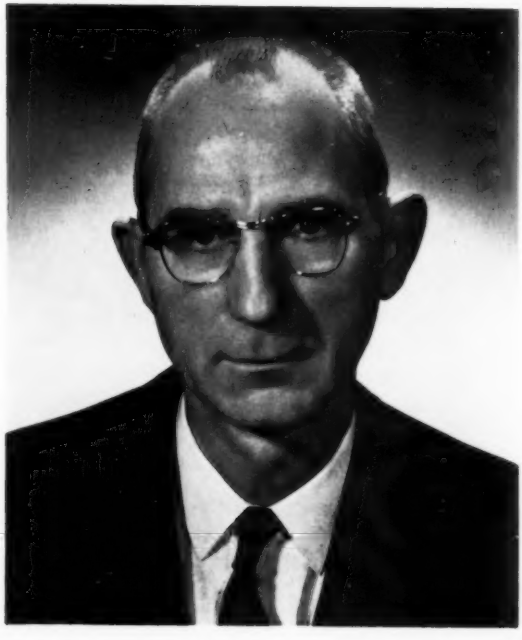
Ross C. MacCardle
1949–1953



Walter E. Heston
1953–1955

Michael B. Shimkin
1955–1960

Howard B. Andervont
1960–

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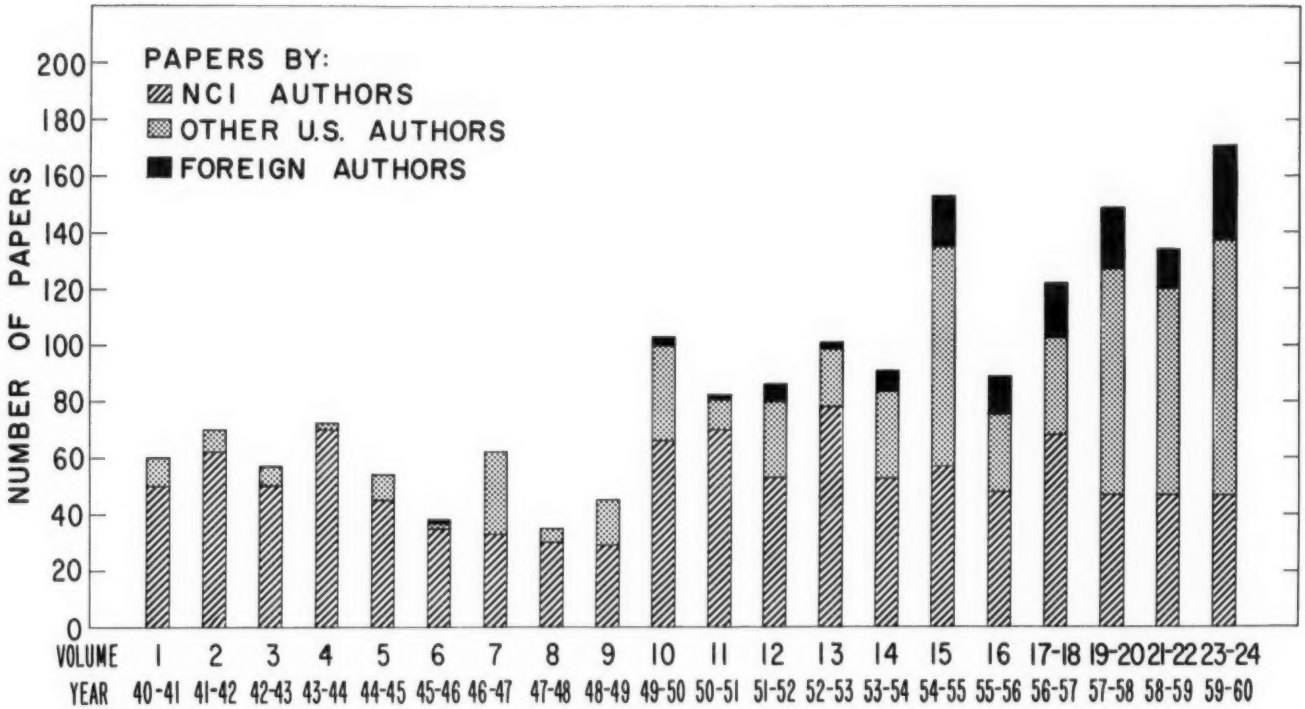

TEXT-FIGURE 1.—Total number of papers in volumes 1 through 24 of the *Journal of the National Cancer Institute*—1940–60.

of all manuscripts of the National Institutes of Health written for publication. At this crucial time, Miss Whitlock retired, Harry Eagle was assigned to another institute, and Howard Andervont asked to be relieved of his long tenure as chairman of the Editorial Committee. To meet Dr. Dyer's recommendation, Dr. Heller established a new Editorial Committee consisting of the Section Chairman (now designated Laboratory or Branch Chiefs) of the National Cancer Institute. As Editor-in-Chief, he called meetings of the Committee to formulate and guide editorial policy. The actual management of the Journal and review of all manuscripts was handled by Ross C. MacCardle, Principal Cytologist, as Scientific Editor.

Dr. MacCardle requested a rotating, working board of scientists at the National Cancer Institute for the double duty of review of manuscripts for the Journal and for other publications. Harlan I. Firminger, Alton Meister, and Katherine Sanford, and later Clifford Grobstein and Lloyd Law, were appointed Assistant Editors. Others who served in this capacity through the years were:

Glenn H. Algire
Carl G. Baker
Morris K. Barrett
Delbert M. Bergenstal
Sanford M. Birnbaum
W. Ray Bryan
Charles C. Congdon
Clyde J. Dawe
William R. Duryee
Thelma B. Dunn
John H. Edgcomb
William S. Fones
Leslie Foulds
James B. Gilbert
Robert E. Greenfield, Jr.
Jonathan L. Hartwell
Albert W. Hilberg
Herbert Kahler
Robert Love
Benno K. Milmore
Eli M. Nadel
Elbert A. Peterson
Walter C. Schneider
Emma Shelton
Jesse L. Steinfeld
Eugene J. Van Scott
Seymour H. Wollman

The Advisory Board was retained, though its members were consulted less frequently, and in October, 1952, this group disbanded. As research in laboratories increased, members of the new Editorial Committee had to devote more time to its direction and were thus retitled Advisers in October, 1953. The working board was named the Board of Editors and by July, 1956, had assumed most of the responsibility for editorial policy and review of National Cancer Institute and Journal manuscripts. Still performing these duties, members of the present Board are: Albert J. Dalton, John L. Fahey, Nathan Mantel, Richard L. Swarm, and John H. Weisburger.

The war had other effects than drastically reducing the size of the Journal. Restrictions on budget, paper, and photographic material had made it most unattractive. Dr. MacCardle was responsible, to a great extent, for revitalizing the Journal and for many developments that led to its expansion. He found officials at the Government Printing Office most cooperative in changing the color, format, and quality of paper of the Journal. The engravers took great pride in improving the photographs and in reproduction of color plates that Dr. MacCardle had obtained permission to publish regularly. He also worked with the research

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staff for better editorial presentation, and invited manuscripts from outside the Institute. His capable assistant was Marjorie Morton, a librarian who served in an outstanding way until Edith E. Parris, of Mallory's laboratory at the Massachusetts General Hospital, was appointed Managing Editor. After the resignation of Miss Parris in 1952, Constance H. Tolkan became Managing Editor and served in that capacity until 1954. Through her efforts, attractive and economical window covers were provided for reprints of articles published in the Journal. The increase in manuscripts and pages (text-fig. 1) undoubtedly reflected these changes.

In October, 1953, Walter E. Heston, Principal Geneticist, accepted the scientific editorship for 2 years. His decision to refrain from further changes until the scientific community had firmly identified the Journal and associated it with the National Cancer Institute proved successful. Volume 15, which included a supplement entitled "Conference on Experimental Hepatomas" and the "Proceedings of the Symposium on 25 Years of Progress in Mammalian Genetics and Cancer," brought the volume to 1,700 pages. There was a significant rise of 63 percent in the number of papers from outside and from foreign countries. The first foreign contribution was 1 paper in volume 6; volume 15 contained 18 articles from other countries (text-fig. 1).

Michael B. Shimkin succeeded Dr. Heston as Scientific Editor in October, 1955. Dr. Shimkin sought to broaden the scope of the Journal by inviting significant speculative, statistical, and clinical research papers. Realizing that the manuscripts published so far were a mere token of those to be expected as a result of expansion in cancer research, in July, 1956, he placed the Journal on a monthly basis so that rapid publication could be maintained. His timing was perfect, as shown by the immediate and continued rise in the number of manuscripts published. Text-figure 1 shows a 50-fold increase in papers from other research institutions in volumes 16 to 24, as compared with the first 5 volumes. Twenty-five percent of these papers in the later volumes were from other countries. Further evidence of outside interest was an award presented in 1959 by the American Medical Writers' Association for accuracy, clarity, conciseness, and newness of information.

Kenneth M. Endicott, who became Director of the National Cancer Institute in July, 1960, appointed Dr. Shimkin Associate Director for Field Studies. Dr. Shimkin retained the scientific editorship until his successor could replace him on January 1, 1961. His stimulating leadership in management, scientific review, and all phases of publication was a valuable asset to the Journal.

Our new Scientific Editor, Howard B. Andervont, has much to recommend him for the post. He is already well known for his contributions to the scientific literature and as former Chief of the Laboratory of Biology. In early years, as chairman of the Editorial Committee, he successfully guided the Journal through a turbulent period. Different problems face us now, and his experience and knowledge will again be needed.

REVIEW AND EDITING OF MANUSCRIPTS

The most important criterion for a manuscript submitted to this Journal is its scientific value. Thus the main responsibility of members of the Board of Editors, who are all active scientists at the National Cancer Institute, is to evaluate the manuscripts on this basis. After a manuscript is assigned to the Associate Editor most familiar with the subject matter, he summarizes it for the Board at the next weekly meeting. Reviewers are then selected, regardless of location, usually on the basis of their participation in the research field dealt with in the paper. It is most gratifying to note how much time is invested by scientists in this arduous task and how seldom a paper is returned without a review. Since volume 16, an acknowledgment recognizing their invaluable assistance has been made in the last issue of each volume.

When all the reviews are in and the Associate Editor has evaluated the reviewers' comments, he again discusses the manuscript with the Board. Pertinent suggestions are presented to the author, and though some may not be valid, they give him an opportunity to consider possible criticisms before the paper is printed.

Over a 10-year period, the percentage of manuscripts rejected has fluctuated between 13.0 and 29.2 percent, with a mean of 21.5.

To expedite this review, an original and two carbon copies, with three sets of illustrations, are requested. If these are received, review can be completed in about a month.

Once approved, the manuscripts are edited by the technical staff and returned to the author so that he will have an opportunity to examine editorial changes before his manuscript is printed.

Though the process seems involved, the average time from date of receipt to publication is 6 months.

WRITING AIDS

Much has been published on this subject and therefore discussion is limited to a few general comments in the hope authors will find them useful as a checklist in submitting manuscripts to this Journal.

Summary: Since many readers now scan the literature, the summary has become an important section of the paper. In recognition of this fact, it was recently placed at the beginning of the article. Summaries from each issue are also given to abstracting journals and compiled in mimeographed form for distribution. For these reasons, the summary should be written so that it is understood without reference to the text. It should include the major points of the paper and they should be stated in specific terms. The summary should preserve the form of the paper, include only material covered in the article, and rarely exceed 250 words. The recent article by Orr and deKoven (*3*) on the summary gives many helpful suggestions.

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Text: Even though there is no arbitrary limit to the number of pages, articles should be concise and well organized.

Illustrations: Black-and-white and color photographs must be essential to the text and should be examined before submittal for accuracy, importance to the paper, composition, and proper focusing. Information given in graphic form should not be repeated in tables.

References: There is a tendency to overuse references. Except for review articles they should be limited to information of definite import to the material in the paper and should not represent an incomplete "review of the literature." Often a review article may be cited if more complete recognition is desired. The correctness of references is considered the responsibility of the author.

Style: Consistency in matters of spelling, abbreviation, and chemical nomenclature are considered important to the over-all appearance of the Journal. Some of the reference material used for such matters include:

Style Manual for Biological Journals, Committee on Form and Style of the Conference of Biological Editors, American Institute of Biological Sciences, Washington, D.C.

Webster's New Collegiate Dictionary, G. & C. Merriam Co., Springfield, Mass.

Dorland's Medical Dictionary, W. B. Saunders Company, Philadelphia, Penna.

Subject Index, Chemical Abstracts, The American Chemical Society, Washington, D.C.

Writer's Guide and Index to English, by P. D. Perrin. Chicago, Scott, Foresman and Co.

A Dictionary of Contemporary American Usage, by B. Evans and C. Evans. New York, Random House, Inc.

A Dictionary of Modern English Usage, by H. W. Fowler. London, Clarendon Press.

Medical Writing, by M. Fishbein. New York, The Blakiston Division, McGraw-Hill Book Company, Inc.

CIRCULATION

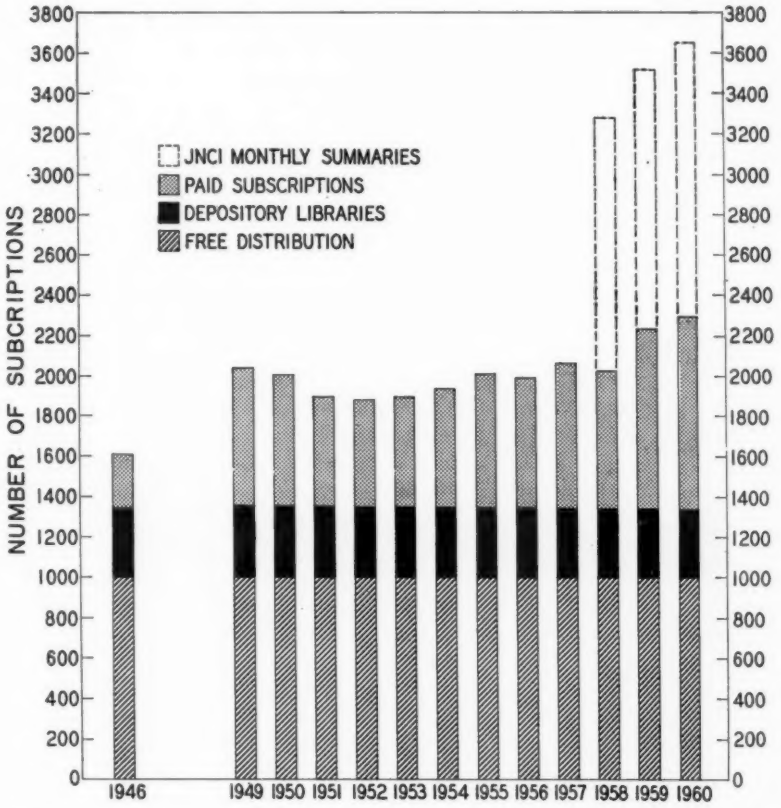
The total circulation of the Journal includes the following categories:

Paid subscriptions: Any scientist or other individual may subscribe to the Journal. Requests for subscriptions should be addressed to the Superintendent of Documents, U.S. Government Printing Office, Washington 25, D.C. The check or money order should be made payable to the Superintendent of Documents, and payment is required in advance. The price is set by the Superintendent of Documents and is now $20.00 for two volumes a year in the United States, Canada, and Mexico; foreign, $25.00. Individual copies may be purchased for $2.00. Data on the number of paid subscriptions in the early years are sparse, but a comparison of 1946 with 1960 in text-figure 2 reveals a fourfold increase.

Depository libraries: Under provision of the law, certain libraries are designated depositories for government publications. Libraries select the publications of interest to their readers, and distribution is made by the Office of the Superintendent of Documents, Government Printing Office. Interested librarians may obtain further information from that office.

Free subscriptions: The National Cancer Institute provides 1,000 copies for free distribution to cancer research departments, institutions, and libraries. Originally, personal subscriptions were given to outstanding scientists and members of the staff. Since 1957, after a review of the problem of accommodating the rise in requests by Ora Marshino, Program Analyst, personal subscriptions have not been offered. To fill the need for personal copies and to explore the usefulness of the Journal in new environments, she established monthly mimeographed summaries. Summaries may be obtained, without charge, by writing to the Journal office.

Text-figures 2 and 3 show the total circulation and the distribution by country. The Journal is now received in each State of the United States and in 77 other countries.



TEXT-FIGURE 2.—Total circulation of the *Journal of the National Cancer Institute*, including summaries.

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TEXT-FIGURE 3.—Distribution of the *Journal of the National Cancer Institute*, by country.

TRENDS

Although the subject matter of material suitable for publication in the Journal remains unaltered, certain trends due to the interests of our contributing authors can be noted through the years. The proportion of articles devoted to certain subjects in volumes 1 through 5 was compared with that of volumes 16 through 24. Some of the more striking changes noted were:

Carcinogenesis was a subject of interest in the first 5 volumes and comprised about 12 percent of all papers. Articles dealt with skin, lung, liver, and subcutaneous tumors and leukemia, which investigators induced by painting and by oral and subcutaneous administration of chemical and physical carcinogens. There has been about a fourfold decrease in papers of this type in the later volumes, though the carcinogens mentioned previously have again received substantial consideration. However, the present trend is toward eliciting information on and gaining an understanding of the mode of action of the carcinogens. Studies on cigarette-smoke condensate have added important information to the epidemiologic reports that have revealed an association between cigarette smoking and lung cancer.

Tumor-virus studies have increased about 20-fold and many types of viruses are now being studied. The mammary-tumor virus and the rabbit-papilloma virus were the only ones reported in the very early volumes; then came studies on the Rous sarcoma virus followed by avian erythroblastosis, myeloblastosis, and lymphomatosis, as well as the Shope fibroma, Friend leukemia, Moloney leukemia, and polyoma viruses, with an upward trend in the latter. There has been an increasing number of papers on electron-microscopy studies of some of these viruses. Certain viruses other than tumor viruses such as lymphocytic choriomeningitis and Coxsackie have been discussed in papers appearing in later volumes.

Leukemia studies have increased, overlapping in certain cases the increase in tumor-virus studies. Two early papers described induced and spontaneous leukemias of mice, and recent reports are on the avian leukemias, transplantable leukemia IRC 741 in rats, and leukemia 2B in guinea pigs. Several papers on the effect of treatment with antimetabolites on the survival of L1210 leukemia, *e.g.*, folic acid antagonists and purine analogues, are in the later volumes.

Biochemical studies comprised about 18 percent of the papers in the first 5 volumes. Many of these were comparative studies on enzymes, proteins, amino acids, nucleoproteins, and vitamins in normal and malignant tissues. There are still many papers of this type. In addition, there are articles describing new methods of analysis, such as differential centrifugation, electrophoresis, chemotherapy, and the ever-increasing use of labeled compounds and microtechniques in studies involving tissue and cell fractions, enzymes, nucleic acids, antimetabolites, and intermediary metabolism of many endogenous and exogenous materials.

Radiation had slight mention in the first 5 volumes, probably because

TEXT-FIGURE 3.—Distribution of the *Journal of the National Cancer Institute*, by country.

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during the war years material on X irradiation was classified. There was a paper on the diagnosis of gastric cancer and a series of papers on experimental roentgen injury; however, a number of papers was published in which ultraviolet radiation was used for tumor induction. In contrast, later volumes contained many papers on X irradiation, but they stressed treatment or the protective capacity of substances against radiation.

Statistical studies, including epidemiology, biometry, and end results, have shown an increasing upward trend. During the first 5 years, the Journal published a total of 6 statistical papers. In one later volume, 1955–56, there were 7 papers, and in 1959–60 the number was augmented to 17. In a comparison of the first and last 5 years, an over-all eightfold increase is evident.

Clinical papers, while still representing a small proportion of the total papers, have risen from 1.2 percent in the first 5 volumes to 5.5 percent in the last 5 volumes. The Journal welcomes clinical studies, but does not customarily accept case reports or simple descriptive articles.

Authorship: In the early years many of the manuscripts were written by the same authors on the staff of the National Cancer Institute. Since it is obvious from text-figure 1 that this is no longer true, we went a step further and compared the number of authors per paper. A rise in the number of authors cited on a manuscript was noted: Of 290 manuscripts in the first 5 volumes 171, or 60 percent, had two or more authors; the remaining 119, or 41 percent, had one author. In volumes 16 through 24, of 664 manuscripts 420, or 73 percent, had two or more authors; the remaining 244, or 27 percent, had one author.

Papers from foreign countries: An interesting trend is the increase in papers from other countries. Contributions have been received from Belgium, Canada, Czechoslovakia, Denmark, Finland, France, Germany, Great Britain, Netherlands, India, Israel, Italy, Japan, Mexico, Peru, Poland, South Africa, Sweden, Switzerland, and USSR. Up to now, all manuscripts have been presented in English. Help is extended to those not familiar with the language and, in some instances, arrangements can be made to translate articles.

NCI MONOGRAPHS

The concern that publishing proceedings, special issues, and commemorative issues might delay publication of research articles jelled to the new idea of starting a National Cancer Institute *Monograph Series*. This series, established in 1959 under Dr. Shimkin's leadership, is an outlet through which scientists may publish, or suggest for publication, material of general interest to cancer investigators, such as groups of papers on oncologic subjects, review articles, and special conferences. All articles are reviewed by the Board of Editors of the *Journal of the National Cancer Institute*, and are indexed in the Journal and in *Index Medicus*.

Four monographs have been published and two more are under way. Since monographs are issued at irregular intervals, they are not available on a subscription basis and must be purchased individually from the

Superintendent of Documents, U.S. Government Printing Office, Washington 25, D.C. A free subscription to the Journal includes a subscription to the monographs. Each new monograph will be announced in the Journal in advance of publication.

REPRINTS

Because of requirements that must be met by authors at the National Cancer Institute, at present 300 reprints are ordered of each article published in the Journal. The reprints are sent to the senior author, without charge, and requests for copies should be addressed to him. Additional reprints may, of course, be purchased by completing the order form sent with the galley proof. The same ruling also applies to the Monographs.

* * *

The Journal has kept abreast of the wave of research manuscripts. Publication in 6 months from date of receipt and 250 pages each month devoted to research papers are good by present standards. The National Cancer Institute *Monographs* are also available for related material. In addition, in the rapidly evolving research field represented by experimental chemotherapy, a special outlet was devised for short and preliminary communications, as well as summaries and other program documents. This is *Cancer Chemotherapy Reports*, issued by the Cancer Chemotherapy National Service Center of the National Cancer Institute. But as in Tennyson's "Choric Song" (*4*)

> Is there any peace
> In ever climbing up the climbing wave?

The concerted effort of cancer investigators all over the world to solve the riddle of cancer and the money and staff now available for their research will undoubtedly produce a greater volume of manuscripts.

If a bimonthly journal was appropriate in 1940 and a monthly in 1956, will the formal scientific journal be adequate for the future? Already a daily biomedical newspaper (*5*) has been suggested. The editors of the *Journal of the National Cancer Institute*, in participation with other interested editorial groups, will share the responsibility for finding practical solutions to problems of scientific communication of the future.

REFERENCES

(*1*) PUBLIC LAW 410: 75th Congress, Chapter 565, 1st Session, Aug. 5, 1937.
(*2*) NCI ARCHIVES: Original justification for the Journal of the National Cancer Institute to the Bureau of the Budget, April 3, 1940.
(*3*) ORR, R. H., and DEKOVEN, D. M.: The summary—the most used and most neglected part of scientific papers. J. Albert Einstein M. Center 8: 270–275, 1960.
(*4*) TENNYSON, A.: The Lotos-Eaters. Choric Song. *In* The Best of Tennyson (Grayham, W., ed.). New York, T. Nelson & Sons, 1930, p. 77.
(*5*) SHIMKIN, M. B.: Science communication for the twentieth century: a daily newspaper of biomedical research. Mississippi Valley M.J. 82: 180–181, 1960.

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Proliferation of Mammalian Cells in Ion-Controlled Environments [1,2]

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SUMMARY

Tissue-culture and nuclei-enumeration procedures were used to study the effects of the aerial net space charge, ion concentration and polarity, on the proliferation rates of mammalian cells *in vitro*. Cells were grown in T-60 flasks containing 15 ml of culture medium, gassed with CO_2 in air, and incubated at 37.5° C. The aerial environment within the culture flasks was altered by a tritium ion generator within the flask. Cultures grown in an atmosphere in which negative ions predominated showed a significantly greater proliferation rate than their accompanying controls, whereas those grown in a positively ionized atmosphere were retarded in proliferation rate to an even more significant degree. A second-transplant generation, though grown in a nonionized atmosphere, tended to retain these altered rates of proliferation originally induced by air ionization. A relationship to similar contemporary physiological experiments is indicated and a possible mechanism for the characteristic biological effects of air ions is cited.—J. Nat. Cancer Inst. 26: 801–811, 1961.

AIR IONS are particles of molecular dimension carrying positive or negative charges. They are produced naturally by solar radiation, radioactive materials, electrical discharges, the friction of movement of masses of air and water, and similar phenomena. Air ions were identified in 1899 (*1–3*) and recognized as possible constituents of a physiologically active component of the atmosphere. Several investigators have devised ion-detecting, measuring, and generating equipment and have sought to relate ion concentration and polarity to physiological response.

Inadequacy of instrumentation caused variability of results in the early investigations, but during the past decade substantially improved ion-detecting and generation equipment has been made available (*4, 5*). As a result, current investigations are highly consistent in experimental findings. Much of the recent research on biological effects of air ions

[1] Received for publication August 30, 1960.

[2] This research was supported by a grant from the Wesix Foundation, San Francisco, Calif.

[3] I wish to thank Raymond E. Roth, Chairman, Department of Mathematics, St. Bonaventure University, formerly Research Associate, Atomic Energy Project, University of Rochester School of Medicine, for statistical evaluation of the data.

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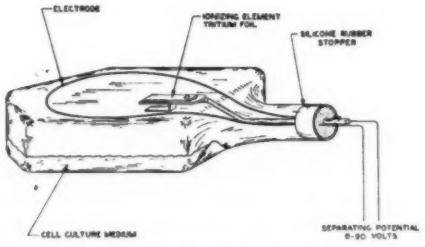
has been carried out with intact organisms, both plant and animal. Studies of growth (*6, 7*), organ response (*8, 9*), activity (*10–12*), and other total parameters have suggested an effect on living things of their ionic environment. Other studies have been concerned with observations at the tissue, cellular, and biochemical levels, and the findings continue to conform to a pattern (*13, 14*). Correlation of the latter aspects with concurrent clinical investigations assumes importance in efforts to determine optimal artificial aerial environments for recuperation, comfort, and survival (*15–20*).

Physiological investigations ranging from the cellular level to that of the entire organism have shown that if positive and negative ions exist in approximately equal numbers in the experimental environment, no physiological response is elicited; yet if there is an excess of ions of one polarity, responses may occur.

The purpose of this investigation has been to confirm and extend data on the relationship of ion concentration and polarity to the growth rate of mammalian cells *in vitro*. The initial phase of this research concerned growth rate of explanted chick blastoderms and of NCTC clone 929 originated from Earle's strain L fibroblasts of the mouse (*13*). In each, the ion source was remote. Blastoderms or cells were cultured in one flask with an interconnecting tubulation to a glass envelope containing the ion generator. Obvious shortcomings of this mechanical arrangement have been eliminated by an ion source that may be inserted directly into the culture flask through its neck (text-fig. 1).



TEXT-FIGURE 1.—T-60 culture flask with tritium ion generator.

This miniaturized ion source employs tritium, a soft beta emitter. Its emissions produce ions of both polarities, but an electrode arrangement removes undesired ions, thus producing a unipolar ionic environment. As used here, 50 mc of tritium, contained in tritiated zirconium on a stainless-steel base, will produce approximately 10^9 ions/second with uniform output. The generation time (mother-cell to daughter-cell anaphase) of clone 929 cells used in this study was consistently longer than usual. Two nutritional restrictions may account for this.

Withdrawal and replacement of nutrient fluid and gassing were impeded by the presence of the ionizing device in the culture flask. Previous

experience indicated the desirability of short-term ion exposure and infrequent feeding.

In anticipation of the determinations herein described, several cultures of cells were slowly transferred from a thrice-weekly to a twice-weekly feeding schedule. After a period of "adjustment" a number of stock cultures of consistent though greatly increased generation time were established. Transplant-generation time was increased from 10 to approximately 14 days.

The second nutritional restriction was dependence on commercially prepared biological fluids. Past use has indicated that successive lots of serum vary the growth rate of cells. At no time has it been possible to maintain a continuously ideal food situation. To eliminate variability, though not prolonged generation time, from this source, quantities of pooled whole-egg ultrafiltrate and of serum, adequate to maintain cultures throughout the entire experimental period, were prepared in advance. The stock of serum was divided into small glass containers and frozen until use. Whole-egg ultrafiltrate was similarly divided and stored at 4° C. Earle's balanced salt solution (BSS) was prepared in quantity in this laboratory, Selas-filtered, gassed, and stored at 10° C.

Girardi's heart cells and culture-medium components were procured commercially. Routine feeding 3 times per week permitted subculturing on the 9th day. Reducing feeding to 2 times per week extended the subculturing time to approximately 16 days.

References in the literature indicate the possibility that filtering may lessen the potency of growth-promoting substances and retard proliferation further.

METHODS

The experimental cells used were NCTC clone 929 fibroblasts (*21*) and Girardi's human heart cells. Girardi's human heart cells and culture-fluid components (except Earle's BSS) were obtained commercially from Microbiological Associates, Inc., Bethesda, Maryland. Parent cultures of clone 929 cells were procured from the Laboratory of Biology, Tissue Culture Section, National Cancer Institute, Bethesda, Maryland.

Clone 929 cells were grown in a medium of whole-egg ultrafiltrate, horse serum, and Earle's balanced salt solution (*22*) in the proportion of 20:40:40. Girardi's heart cells were cultured in human serum, Eagle's basal medium, and L-glutamine, 5:95:1. T-60 flasks (tissue-culture glassware and apparatus from Schaar and Co., 7300 W. Montrose Ave., Chicago 34, Ill.) were employed for both stock and test cultures. Fluid was replenished twice per week, flasks were gassed with 5 percent CO_2 in air, and incubation was at 37.5° C. Incubator temperatures were recorded continuously.

Culture medium was freshly prepared at the time of each fluid replacement and pressure-filtered (015 Selas filter, 5 lbs. pressure, humidified 5 percent CO_2 in air) immediately before being dispensed. No antibiotic

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was used. Hydrogen ion concentration of gassed medium was determined at the time of feeding and that of spent medium at the time of withdrawal from the culture flasks.

The *p*H of medium at the time of dispensing ranged from 7.4 to 7.45. The maximum range of *p*H of spent fluid was 6.9 to 7.2, but 6.95 and 7.0 recurred more frequently. There was no evident relationship between the *p*H of spent fluid and the ion polarity.

At each fluid change, 10 of the 15 ml of spent culture medium was replaced with freshly prepared medium. During operations, such as subculturing, washing, replicating, and other handling of cells, freshly prepared, whole culture medium was used. The use of one component, such as balanced salt solution, for this purpose was inexpedient.

The tritium ion generator was integral with the silicone flask stopper. It was inserted after fluid changes, and while in operation was connected to a 90 v d-c source. The possibility of this separating potential exerting an independent physiological effect has been investigated and discounted by Krueger (*14*) and others.

A vigorous, healthy colony of cells was selected for each determination of proliferation rate. The cell sheet was rinsed with whole culture medium, cells were removed by agitation in 45 ml of culture fluid, sieved through a platinum screen, and dispensed from a mixing apparatus into 3 aliquots (*23*). One portion was set up for ionization, one as a nonionized accompanying control, and the third used for nuclear enumeration (*24*). This study was restricted to initial populations of 2 million cells, ± 20 percent, per 15 ml of culture fluid in view of the known relation of inoculum size to proliferation rate (*25*). If the nuclear enumeration indicated that the inoculum exceeded these limits, the subcultures were returned to stock; this accounts for the narrow range of initial populations (1.5 to 2.3 million) as indicated in tables 1 and 2.

Cultures were subjected to ionization or control conditions, fed and gassed twice weekly, and at the 4th feeding (14 days) each was diluted to 30 ml and divided into 2 aliquots, 1 for continued incubation and 1 for enumeration.

During the second-transplant generation, cells previously ionized were maintained without ionization and under the same conditions as the controls. The first-transplant generation served to indicate the possible immediate effects of ionization, and the second, to observe any continued effects of the ionization experience. Feeding continued in the same way, and, at the end of the second 14-day period, cultures were terminated for nuclear enumeration.

RESULTS

Clone 929 Cells

Tables 1 and 2 show cell populations at the beginning and end of each experimental period. Comparison of columns 2 and 3 shows the change

in number of cells during the 14-day ionization period, and of columns 4 and 5, the change during the following 14 days with no ionization. The population of each culture's accompanying control is shown. Proliferation factors (final population : initial population) are noted in parentheses in each instance.

TABLE 1.—Proliferation of NCTC clone 929 fibroblasts. First-transplant generation, 14 days, with negative ionization, and accompanying controls with no ionization. Second-transplant generation, 14 days, with no further ionization and accompanying controls with no ionization

Subculture identification	First-transplant generation. No. of cells ($\times 10^6$)	Number of cells after 14 days of incubation ($\times 10^6$)		Second-transplant generation. No. of cells ($\times 10^6$, ½ col. 3)*	Number of cells after 14 days of incubation ($\times 10^6$)	
		Negative ionization	Controls, no ionization		Previously ionized	Controls, no ionization
N-1, negative	1. 821	3. 450 (1. 89)†		1. 725	3. 998 (2. 32)	
N-1, control	1. 821		3. 350 (1. 84)	1. 675		3. 800 (2. 27)
N-2, negative	1. 650	3. 385 (2. 05)		1. 692	3. 700 (2. 19)	
N-2, control	1. 650		3. 035 (1. 84)	1. 517		2. 500 (1. 65)
N-3, negative	2. 300	4. 371 (1. 90)		2. 185	4. 575 (2. 09)	
N-3, control	2. 300		4. 200 (1. 83)	2. 100		4. 005 (1. 91)
N-4, negative	2. 100	3. 899 (1. 86)		1. 949	3. 101 (1. 59)	
N-4, control	2. 100		4. 032 (1. 92)	2. 016		2. 995 (1. 49)
N-5, negative	1. 630	3. 215 (1. 97)		1. 607	3. 831 (2. 38)	
N-5, control	1. 630		3. 000 (1. 56)	1. 500		2. 500 (1. 67)
N-6, negative	1. 922	3. 989 (2. 08)		1. 994	3. 511 (1. 76)	
N-6, control	1. 922		3. 756 (1. 95)	1. 878		3. 107 (1. 65)
Means Negative	1. 904	3. 885 (1. 96)		1. 942	3. 786 (2. 05)	
Means Control	1. 904		3. 562 (1. 82)	1. 781		3. 151 (1. 77)

*Remaining half used for nuclear enumeration.

†Figures in parentheses are the proliferation factors, *i.e.*, ratio of final population to the corresponding initial population.

Cultures, while under the influence of ionization, proliferated at rates differing from their controls—the negatively ionized cells were accelerated slightly, and the positively ionized cells were decelerated greatly. During the ensuing 14-day period with no ionization those cells previously exposed to negative ions continued to proliferate at a slightly increased rate as compared to their controls. However, the previously positively

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TABLE 2.—Proliferation of NCTC clone 929 fibroblasts. First-transplant generation, 14 days, with positive ionization, and accompanying controls with no ionization. Second-transplant generation, 14 days, with no further ionization and accompanying controls with no ionization

Subculture identification	First-transplant generation. No. of cells at start (× 10^6)	Number of cells after 14 days of incubation (× 10^6)		Second-transplant generation. No. of cells (× 10^6, ½ col. 3)*	Number of cells after 14 days of incubation (× 10^6)	
		Positive ionization	Controls, no ionization		Previously ionized	Controls, no ionization
P-1, positive	1. 551	1. 587 (1. 02)†		. 793	. 750 (0. 96)	
P-1, control	1. 551		3. 107 (2. 00)	1. 553		3. 100 (2. 00)
P-2, positive	1. 633	2. 216 (1. 33)		1. 108	2. 000 (1. 81)	
P-2, control	1. 633		2. 895 (1. 77)	1. 447		3. 450 (2. 38)
P-3, positive	1. 883	1. 000 (0. 53)		. 500	No count	
P-3, control	1. 883		3. 876 (2. 06)	1. 938		3. 023 (1. 56)
P-4, positive	2. 035	2. 100 (1. 03)		1. 050	1. 000 (?) (0. 95)	
P-4, control	2. 035		2. 799 (1. 38)	1. 399		2. 133 (1. 52)
P-5, positive	1. 718	1. 565 (0. 91)		. 782	1. 331 (1. 70)	
P-5, control	1. 718		3. 400 (1. 98)	1. 700		2. 905 (1. 71)
P-6, positive	2. 252	2. 357 (1. 05)		1. 178	1. 958 (1. 66)	
P-6, control	2. 252		4. 175 (1. 85)	2. 087		3. 987 (1. 91)
Means Positive	1. 845	1. 604 (0. 98)		. 802	1. 408‡ (1. 42)	
Means Control	1. 845		3. 375 (1. 84)	1. 687		3. 099 (1. 85)

*Remaining half used for nuclear enumeration.

†Figures in parentheses are the proliferation factors, *i.e.*, ratio of final population to the corresponding initial population.

‡N=5.

ionized cells, though showing a slight increase in their rate of proliferation, were still below that of their entirely nonionized controls.

Gross and microscopic examination of clone 929 cells revealed the following:

1) Negatively ionized and control cultures became established on the glass substrate and proliferated in a characteristic and typical manner. Cells were scattered more or less evenly over the flask bottom, singly or in loose aggregates, 24 hours after subculturing. Flattened, slim spindle shapes predominated, with a few triangular and multipolar forms. As the cells proliferated and formed a macroscopically visible sheet, microscopic examination showed an ever-closing, meshlike arrangement of the cells—their long processes in close proximity, overlapping or apparently

adherent. Orientation was random. As cells became somewhat crowded toward the end of each transplant generation, rounding, then spherical, discreet cells predominated. The light, delicately granular cytoplasm of the young cells was replaced by a more darkly granular variety. There were no discernible differences, other than revealed by nuclear enumeration, between control and negatively ionized cells.

2) Positively ionized cultures seemed to resist adhesion to the glass. Some cells would establish themselves and begin to proliferate in a normal manner. The sheets formed were generally thin and sparse, and the rounded, more granular forms appeared early, before crowding became evident. Many cells proliferated in suspension and tended to be spherical in form. In some cultures, cells once adherent later loosened and could be dislodged readily by gentle agitation of the fluid in the flask. During the second-transplant generation, without ionization, conditions approached normalcy but still could be thus chacterized. Some that survived the positive ionization experience were continued through several subcultures and ultimately attained a characteristic proliferation pattern.

Girardi's Human Heart Cells

Cultures of Girardi's human heart cells were subjected to conditions of care, preparation, and ionization identical to those described for clone 929 cells. Gross and microscopic examination of heart-cell cultures showed that response was similar to that of clone 929 cells. The technique of nuclear enumeration, as here used, presented difficulties to an extent that the accuracy of the counts was doubtful and their compilation as data would be unwarranted.

Subsequently each of 8 stock cultures was divided into 3 portions. One was prepared for negative ionization, 1 for positive ionization, and the 3d as a common nonionized control. The same general procedures were followed as with clone 929 cells, except for deletion of the nuclear count.

Gross and microscopic examination of Girardi's human heart cells indicated the following:

1) Negatively ionized and control cultures appeared to grow identically during both intervals—0 to 14 days and 14 to 28 days—except that during the second-transplant generation 2 previously ionized cultures perished. Observations were made 8 times during this 28-day period, and at no time were there greater differences among the ionized and control cultures than one would ordinarily observe among any group of similar cultures.

2) Positively ionized cells showed a variety of divergences in rate and manner of growth from that of the controls. Of the original 8 cultures, 3 became normally adherent to the flask floor, proliferated slowly, and at no time reached a cell density near that of the controls. During the second generation, with no ionization, these same cultures continued to

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grow normally, proliferated more rapidly than during the period of ionization, but again failed to keep pace with their controls.

Of the 8 positive cultures, 2 formed scant, discontinuous sheets of loosely adherent cells with many cells in suspension. Second-transplant-generation growth was similarly scant and atypical.

The remaining 3 positive cultures did not survive the ionization experience. One showed only cellular debris at the 7th day, 2 survived through the 10th day, but no viable cells could be found on the 14th day.

Statistical Evaluation

Clone 929 cells.—1) Application of the F test for significant differences of variances of the means of the proliferation factors for the nonionized controls of both negatively and positively ionized groups, for both generations, indicates consistency of growth rate. This is shown in table 3. The values obtained demonstrate that there is no significant difference of variance and validate the application of Student's *t* test in the comparison of control with corresponding ionization means. The sample variance used in each was a pooled estimate for the samples compared (*26*).

TABLE 3.—F test for significant differences of variances. Values below 5.05 indicate that there is no significant difference of variance. The values shown in this table justify the use of Student's *t* test for differences of means*

Days	Negative versus control	Positive versus control
At 14	2. 33	1. 09
14–28	1. 30	1. 73

*See table 4.

2) Student's *t* test showed that, in comparison of the proliferation rates of controls with ionized cultures, there was a significant difference of means for each period of time and for each condition of ionization. Each value shown in table 4 represents a significant difference at the 95 percent level. Correlating this with the data of tables 1 and 2 shows that negative ions promote proliferation and that positive ions retard proliferation of clone 929 cells, and that the effect is carried over into the following nonionized generation.

DISCUSSION

The data show, with statistical significance, that proliferation rate of clone 929 cells was accelerated when subjected to an atmosphere rich in negative ions; that proliferation was more markedly decelerated under conditions of positive ionization; and that the ionization experience had some continued effect on later proliferation of these cells. Negatively ionized cells continued to proliferate at an increased rate. Positively

TABLE 4.—Student's *t* test for differences of means. A value of 2.228 or greater shows significant difference at the 95 percent level, where N equals 6. Where N equals 5 (table 2) this value is 2.262

	t-Calculated		
Days	Negative ionized vs. negative controls	Positive ionized vs. positive controls	Negative controls vs. positive controls
0–14	4. 22	13. 1	Less than 1*
14–28	3. 32	4. 44	Less than 1

*No significant difference.

ionized cells proliferated a little more rapidly after ionization was terminated but did not attain a preionization rate. Their lack of recovery may be attributed to the unfavorably small number of cells in each subculture at the beginning of the last generation as well as to carry-over effect from ionization.

Girardi's human heart cells grew normally under conditions of negative ionization, as evidenced by comparison with accompanying controls. Positive ionization had a markedly adverse effect on growth habit and rate of proliferation.

These data are compatible with the studies of Krueger and Smith on functional changes in mammalian trachea brought about by atmospheric ions. They found that positive ions inhibited the normal action of the tracheal-clearing mechanism and induced a state of increased vulnerability to trauma in cilia and subadjacent blood vessels. Negative ions reversed most of these effects and increased the rate of ciliary beat to levels well above normal (*27–30*). Krueger and Smith have concluded that negatively ionized oxygen and positively ionized carbon dioxide are the components of ionized air which are responsible for the various biological effects ascribed to air ions (*31*). As a result of their experiments with tissue homogenates *in vitro* (*14*), and the neurohormone, 5-hydroxytryptamine (5-HT) *in vivo* (*32*), Krueger and Smith suggested a mechanism by which ionized oxygen and carbon dioxide may produce their characteristic effects, as follows: Carbon dioxide in the positively ionized state appears to mediate the release of 5-HT, which subsequently accumulates locally in tissues so affected. Negatively ionized oxygen reverses this effect by increasing the rate of oxidation of the free 5-HT. The specific enzyme involved, monamine oxidase, is thought to consist of a dehydrogenase linked to a respiratory chain which may include cytochromes or flavines.

Krueger and Smith have further shown that negative ions have an effect on cytochrome oxidase and accelerate the cytochrome-linked conversion of succinate to fumarate. These authors suggest that this same effective of negative ions could be responsible for acceleration of a cytochrome-linked oxidation of 5-HT.

Although applicability of the conclusions of Krueger and Smith to the work herein reported is not immediately apparent, there is a distinct parallelism between the results of their physiological experiments and those reported here. In each case certain activities of mammalian cells were depressed or inhibited by positive ions and accelerated by negative ions. The similarity extends to duration of ion action as well, for in each instance there was observed a "carry over" of ion effect after discontinuing exposure to ionized atmospheres (*33*).

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Strain Differences in Growth of Mouse Mammary Tumors [1, 2]

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SUMMARY

Four hundred female mice of strains C3H, BALB/c_t, and RIII, all susceptible to the development of mammary tumors, bearing the milk agent, and endowed with physiologic hormonal stimulations (normal breeding), have been studied. The analysis concerns the rate of fertility, tumor incidence, tumor age, survival of tumor-bearing mice, occurrence of multiple tumors, growth rate of tumors, frequency of partial regressions, and type of tumor growth. Under the same conditions, C3H and BALB/c_t mice possess a nearly parallel behavior, but in RIII mice the behavior of tumors is quite different. The tumors occurring in the two former strains possess a high growth rate, rarely undergo partial regressions, and grow in regular types, mostly a sigmoidal curve. In contrast, tumors occurring in the RIII strain possess a low growth rate, frequently undergo partial regressions, and develop irregular and unpredictable types of growth. Possible causes for these differences are suggested.—J. Nat. Cancer Inst. 26: 813–828, 1961.

THE PRACTICE of inbreeding has led to the isolation of a number of inbred strains of mice. Some are resistant and some susceptible to the development of mammary tumors. The latter may be either low-cancer strains or high-cancer strains. Mammary tumors of high-cancer-strain mice are caused by the penetration with milk of a virus-like particle, or mammary-tumor agent, into susceptible hosts endowed with physiologic hormonal stimulations (estrous cycles, pseudopregnancies, and pregnancies).

The incidence and age at onset of these tumors vary in different strains. Moreover, experience with tumor-bearing mice shows that the tumor growth is also a variable characteristic in some way related to the mouse strain. The tumor incidence and tumor age measure both the intensity of the etiologic factors and the genetic susceptibility of the host tissues. Yet the growth of tumors, as it is expressed by conventional growth

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curves, measures the characteristics, composition, and behavior of the tumor-cell populations and gives information on the growth potential of the cells, their dependence or independence on extrinsic stimuli, the times of their progression toward increasing autonomy, the rules of their multiplication *in vivo*, etc.

Although the general problems connected with the growth of tumors have been extensively investigated (*1–7*), very few contributions are available in the literature concerning the growth of spontaneous mammary tumors in inbred mice (*8, 9*). In the past some attempts were made with tumor-bearing stock mice (*10–14*), and recently a study has been devoted to hybrid lines showing peculiar characteristics in the growth curves of tumors (*15*). This paper deals mainly with the characteristics of tumor growth in three different high-cancer-strain mice of common use for experimental work: C3H, BALB/c_t, and RIII.

MATERIALS AND METHODS

The investigation has been carried out on 400 breeding female mice of the C3H, BALB/c_t, and RIII strains. The C3H strain was sent from the University of Minnesota, Minneapolis (J. J. Bittner), to the Chester Beatty Research Institute, London (A. Haddow), at the 78th inbred generation, and from the Chester Beatty Research Institute to the Division of Cancer Research, Perugia (L. Severi), November 6, 1953, at the 83d inbred generation; now it has reached the 100th inbred generation (C3H/Cb/Se substrain). The BALB/c_t strain is BALB/c foster-nursed by C3H. This strain was sent from the Cancer School of Medicine, Leeds (L. Dmochowski), to the Chester Beatty Research Institute, London, at the 36th inbred generation, and from the Chester Beatty Research Institute to the Division of Cancer Research, Perugia, November 6, 1953, at the 40th inbred generation; now it has reached the 63d inbred generation (BALB/c_tC3H/Cb/Se substrain). The RIII strain was sent from Columbia University, New York (L. Dmochowski), to the Division of Cancer Research, Perugia, August 16, 1954, at the 12th inbred generation; now it has reached the 29th inbred generation (RIII/Dm/Se substrain). The animals used in this study belong, respectively, to the following inbred generations: F_{91} to F_{95}, C3H; F_{50} to F_{61}, BALB/c_t; and F_{19} to F_{28}, RIII.

After weaning, the female mice were mated. All animals were kept in metallic cages, 2 females and 1 male in each cage. When both females were pregnant, one was separated from the male and all were permitted to lactate their newborns. The litters were weaned when 25 to 30 days old, and then the mothers which had left their original cage were replaced by males.

A diet of cubes and water *ad libitum* was given. The cubes, supplied by Pilsbury's, Ltd., Birmingham, England, were prepared with the following ingredients: wheat, oats, barley meal, maize, meat and bone meal, dried

milk, fish meal, yeast, molasses, salt, and cod-liver oil. The diet was constant for all mice throughout the experimental period.

The animals were observed until death. Records were kept of the reproductive histories of each mouse and the occurrence of all palpable mammary tumors. A total of 591 palpable growths was detected in 342 tumor-bearing mice. Once a week each tumor was measured with calipers in the 2 largest diameters, and the average of these values was used as a weekly measure of the neoplasia. The average tumor growth per week was estimated by division of the difference between average final and average initial tumor diameters by the number of weeks of survival. If stages of partial regression occurred during the growth of tumors, they were not separately handled.

For studies on the fertility rate, tumor incidence, tumor age, survival time, and occurrence of multiple tumors, all animals were used, with the only exceptions specified later. But in the analysis of the characteristics of tumor growth, subdivision of the material was needful, since pregnancies are known to influence the growth of mammary tumors in some lines of mice (*15*). Therefore, in each strain the tumor-bearing mice have been divided into 2 series: those in which no deliveries occurred during the palpable stage of tumor growth (Series I), and those in which deliveries occurred (Series II). Due to the striking differences in the rate of fertility and in the clinical duration of tumors, the distribution of mice into the 2 series appears to be uneven in the three strains, Series I containing 50 C3H mice, or 94 percent, 133 BALB/c_f mice, or 75 percent, and 58 RIII mice, or 52 percent. The growth of tumors in mice of Series II will be discussed in a later paper.

Some of the animals were allowed to die spontaneously, others were killed at various stages of tumor development for histologic purposes, but most were killed when moribund. In the calculation or the average survival of tumor-bearing mice, and the other data which may be influenced by survival, only the first and last groups of animals were used. These include 49 female mice of the C3H strain, 122 of the BALB/c_f strain, and 63 of the RIII strain. All animals were autopsied and the tumors removed for histologic examination.

RESULTS

General Features of Mammary Tumorigenesis in C3H, BALB/c_f, and RIII Strains

In table 1 the tumor incidence and age, survival time, and occurrence of multiple tumors in breeding females of the C3H, BALB/c_f and RIII strains are shown. In addition, some information on the causative factors of tumors is given, including the sources of the respective milk agents and the average rates of fertility.

TABLE 1.—General features of mammary tumorigenesis in breeding female mice of the C3H, BALB/c_t, and RIII strains

Strain	Source of milk agent	Average No. of litters per mouse	Number of mice	With mammary tumors		Average age of tumors (days)	Average survival time*† (days)	Average No. of tumors per mouse*	Without tumors	
				Number	Percent				Number	Average age at death (days)
1	2	3	4	5	6	7	8	9	10	11
C3H	C3H	1.2	58	53	91.4	320	49	1.6	5	486
BALB/c_t	C3H	2.5	190	177	93.2	277	59	2.2	13	423
RIII	RIII	3.7	152	112	73.7	301	103	1.5	40	455
Total		2.8	400	342	85.5	292	72	1.9	58	450

*These data concern 49 females of the C3H strain, 122 of the BALB/c_t strain, and 63 of the RIII strain that died a natural death or were killed when moribund
†From the appearance of a palpable mammary tumor to the death of the animal.

Origin of Strains and Sources of Milk Agent

The strains investigated have a different genetic origin. The C3H was obtained by mating a DBA male to a Bagg albino female, and the BALB/c is a continuation of one of the branches of the original Bagg albino mice, whereas the RIII, which is also an albino strain, originated in Paris (*16*). All these strains bear the mammary-tumor agent. The C3H and RIII strains have possessed a milk agent since their origin, but in BALB/c_f mice the agent was transferred by foster nursing from C3H mothers. Therefore, the C3H and BALB/c_f strains derive their milk agent from the same source (C3H), while the RIII strain derives it from another (RIII).

Fertility Rate

The average number of deliveries in mice of the 3 strains is illustrated in column 3 of table 1. The C3H mice, though having an average lifespan approximately equal to that of RIII mice and longer than that of BALB/c_f mice, show the lowest fertility rate, 1.2 litters per mouse, compared with 2.5 in BALB/c_f and 3.7 in RIII mice.

Tumor Incidence and Tumor Age

Of the C3H mice, 91.4 percent developed one or more mammary tumors at an average age of 320 days, compared with 93.2 percent of the BALB/c_f mice and 73.7 percent of RIII mice that grew tumors at an average age of 277 and 301 days, respectively (table 1, cols. 5–7). Usually it is assumed that the higher the tumor incidence, the earlier the tumor age, but this assumption does not agree with the values found in C3H mice, in which the high percentage of tumors was associated with the latest tumor age.

Survival Time

Survival time is defined as the time elapsing from the appearance of a palpable mammary tumor to the death of the animal. Column 8 of table 1 shows the average survival of tumor-bearing mice. Only the animals that died a natural death or were killed when moribund are considered. The C3H mice had the shortest survival, 49 days, and the RIII the longest one, 103 days. BALB/c_f mice showed a survival time of 59 days.

Multiplicity of Tumors

The occurrence of multiple tumors in the mammary-gland system of single hosts is a common event in high-cancer-strain mice. Also when the animals develop a single palpable growth, other mammary tumors of submacroscopic size and premalignant changes are often found at autopsy or during the histologic examination of the glands (*8*, *17*). In column 9 of table 1 the average number of tumors per mouse in C3H, BALB/c_f, and RIII strains is given. Animals killed before they were

actually moribund were discarded, and only the palpable tumors have been considered. The BALB/c_t strain shows the highest average value, equal to 2.2 tumors per mouse, followed by the C3H with 1.6 and the RIII with 1.5. Since the development of secondary tumors depends also on the time which they have to develop, the differences in the average number of tumors per mouse may be larger than they appear because of the differences in survival times shown by the 3 strains of mice.

Characteristics of Tumor Growth

Growth Rate of Tumors

The rates of tumor growth in mice of the C3H, BALB/c_t, and RIII strains are illustrated in table 2. Only 405 tumors, in 241 female mice which did not deliver litters during tumor growth (Series I), are considered, to avoid the possible influence of extrinsic factors. Primary and secondary growths were mixed. In the table, the tumors have been divided according to their average speed of growth per week, and the total mean value is given. In all strains the neoplasms were discovered, on average, when measuring 0.6 to 0.7 cm in the mean diameter. The average rate of tumor growth is approximately the same in C3H and BALB/c_t mice, ranging from 0.238 to 0.231 cm per week ($P > 0.2$), whereas it is markedly lower, 0.135 cm per week, in tumors of the RIII strain. Accordingly, the difference between the averages shown by the former and latter strain is highly significant ($P < 0.001$).

In the RIII strain, 75.7 percent of the tumors showed an increase of their mean diameter equal to or less than 0.2 cm per week, compared with 41.9 and 41.7 percent in C3H and BALB/c_t strains, respectively. Therefore, in each strain there is presented by the majority of tumors a prevalent speed of tumor growth, which is lower in the RIII strain (≤ 0.2 cm per week) and higher in the C3H and BALB/c_t strains (> 0.2 cm per week). Nevertheless, a few RIII tumors exceed the slow growth rate prevalent in this strain, and some C3H and BALB/c_t tumors do not reach the rapid growth rate. Therefore, despite the genetic purity of inbred mice, the speed of growth for single spontaneous tumors within each strain remains an unconstant and unpredictable characteristic, and the problem may be solved at present only in terms of prevalence. Because of this and the relative infrequency of linear growths in tumors, the average growth rate which characterizes the various strains must be regarded as a theoretical, though indicative, value. A higher stability in the growth rate of single tumors may be attained, however, by repeated transplantations.

Factors Affecting the Growth Rate of Tumors

The growth of mammary tumors in mice may be influenced by various coincidental factors such as the reproductive activity of the host (*12*, *15*), the multiplicity of tumors, their location within the mammary-gland

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TABLE 2.—Growth rates of mammary tumors in breeding female mice of the C3H, BALB/c$_f$, and RIII strains

Strain	Number of tumor-bearing mice	Number of palpable mammary tumors*	Average initial tumor size (cm)	Tumors divided according to their average growth in cm per week														Average tumor growth per week (cm)	Standard deviation	
				Stationary or regressing		Progressing														
						0.001 to 0.100		0.101 to 0.200		0.201 to 0.300		0.301 to 0.400		0.401 to 0.500		0.501 to 0.600		0.601 to 0.700		
				Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent			
C3H	50	76	0.6	1	1.3	5	6.7	25	33.8	24	32.4	12	16.2	5	6.7	1	1.3	1	1.3	0.238	0.123	—
BALB/c$_f$	133	254	0.7	4	1.6	26	10.5	73	29.5	84	34.0	37	15.0	17	6.9	6	2.4	—	—	0.231	0.121	1.16†
RIII	58	75	0.6	4	5.4	29	39.2	23	31.1	10	13.5	8	10.8	—	—	—	—	—	—	0.135	0.108	10.3‡
Total	241	405	0.7	9	2.3	60	15.2	121	30.6	118	29.9	57	14.4	22	5.6	7	1.8	1	0.2	0.214	0.125	

*Ten of these tumors: 2 of the C3H strain, 7 of the BALB/c$_f$ strain, and 1 of the RIII strain, were discarded, having been measured once only.
†$0.3 > P > 0.2$.
‡$P < 0.001$.

system, the occurrence of necrosis, hemorrhages, or infections in tumors (*13*). Moreover, relations seem to exist also between the histologic architecture and rate of tumor growth (*18*). The question of the possible influences exerted by pregnancy on the growth of mammary tumors, as well as that of the relationships between growth and morphology, which have resumed importance and actuality since the works of Foulds (*15*, *18–22*), will be analyzed in further papers. A brief account on the other minor questions in the present material will be given here.

Single and multiple mammary tumors have been grouped separately, and the respective average growths per week calculated. The single tumors grow more than multiple tumors, but the difference between the two groups, though constant in all strains, is slight (0.015 cm per week) and not significant ($P > 0.2$). The rate of tumor growth does not show significant differences even in relation to the fore or hind site of tumors ($P > 0.8$). This observation contrasts with previous results obtained in tumor-bearing stock mice (*13*), in which a significant difference was found between the growth rates of thoracic-cervical and abdominal-inguinal tumors, the latter growing more than the former.

The purulent infection of nonulcerated tumors was infrequent in the present material, whereas the occurrence of necrosis and hemorrhages was often observed in neoplasms. These accidents, however, do not seem to interfere greatly with the growth speed of tumors during the early stages of development. Moreover, they seem unrelated to the decrease of growth-speed characteristics of the latest tumor stages that occur even in ascites tumors, in which the cells do not undergo any regressive change (*7*).

Regressions of Tumors

Total regressions of palpable tumors have never been observed in this study, whereas stops of growth and partial regressions of variable degree and duration were frequently seen. Although some of these tumor regressions may have been due to inadequacies of the method of measurement, their uneven distribution into the 3 tumor strains investigated here deserves more detailed information. Of the RIII tumors, 59 percent have shown one or more stages of regression during their clinical course, compared with 34 and 27 percent of the BALB/c_t and C3H tumors, respectively. The chi-square test denotes a significant association between the RIII strain and the regressions in tumors ($P < 0.001$). Moreover, in RIII mice stops and regressions may repeatedly occur in the same tumor, whereas in the other strains they usually appear as isolated and accidental. In other words, the stops and the partial regressions during the growth of tumors are an almost constant feature in the RIII strain, whereas they seem to be occasional exceptions in the other strains of mice.

Growth Curves of Tumors

The exponential curve has been repeatedly assumed to be a theoretical model of the multiplication of tumor cells *in vivo* (*3*, *23*), but the experi-

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mental data were seldom found to fit with this expected type of curve (*3, 4, 13*). An explanation of this fact was given for solid tumors, based on the observation that only the peripheral cells proliferate, while the central mass undergoes regressive changes (*3*). However, the exponential law of growth was found unfitting also in ascites tumors, where the "central" necrosis may not occur (*7*). Linear growth is the most frequent to be observed in some transplanted tumors of the rat (*3–6*), whereas the growth of mammary tumors transplanted in mice is usually of a sigmoidal shape, *i.e.*, an initial phase of slow growth, followed by a rapid growth phase and ending with a slow phase again near the spontaneous death of the host (*24*). A somewhat similar behavior is also characteristic of the growth of Ehrlich ascites tumors, in which three successive phases have been recognized by means of cell-counting methods: latency period, cube-root phase, and terminal period. The rate of multiplication of free tumor cells in the peritoneal fluid is slow during the first and third periods, but it is rapid and increases linearly in the third power of time during the second period (*7*). The reduction of growth during the third stage of tumors seems unrelated to regressive cell changes.

Spontaneous mammary tumors of mice have not been satisfactorily investigated in this respect. Sometimes they develop according to a straight line, but other types of growth may be observed (*13, 9*). Representative samples of the growth curves of tumors occurring in C3H, BALB/c_t, and RIII strains are illustrated, respectively, in text-figures 1, 2, and 3. It is apparent that there is a variety of types of growth, such as sigmoidal, linear, parabolic, and exponential. Moreover, a number of tumors, mainly grouped in the RIII strain, do not fall in any of those recognizable model curves because of recurring stops and regressions which make their trends irregular. This type of growth will be referred to as "irregular," in contraposition to the other types.

In table 3 all the tumors showing classifiable trends have been divided in groups according to their type of growth. Analysis of the totals indicates that tumors with regular growth curves overcome the tumors showing irregular growths, and that, among the first group, there is a prevalence of sigmoidal and linear growths, whereas the parabolic are infrequent, and the exponential rare. When, however, the figures are analyzed separately for each strain, it becomes apparent that in C3H and BALB/c_t mice about two thirds of the tumors show regular growth curves, compared with less than one third in RIII mice. If the data observed in BALB/c_t and RIII mice are compared separately with those of the C3H strain by means of the two-by-two contingency table, an insignificant ($P > 0.7$) and a highly significant ($P < 0.001$) chi-square value is found. This is another difference which emphasizes the peculiar biology of neoplasms of the RIII strain.

The linear, parabolic, or exponential types of growth may approach, with some approximation, a sigmoidal curve, of which they would represent incomplete portions. Therefore, it seems reasonable to take this curve as the theoretical model of development for mammary tumors in

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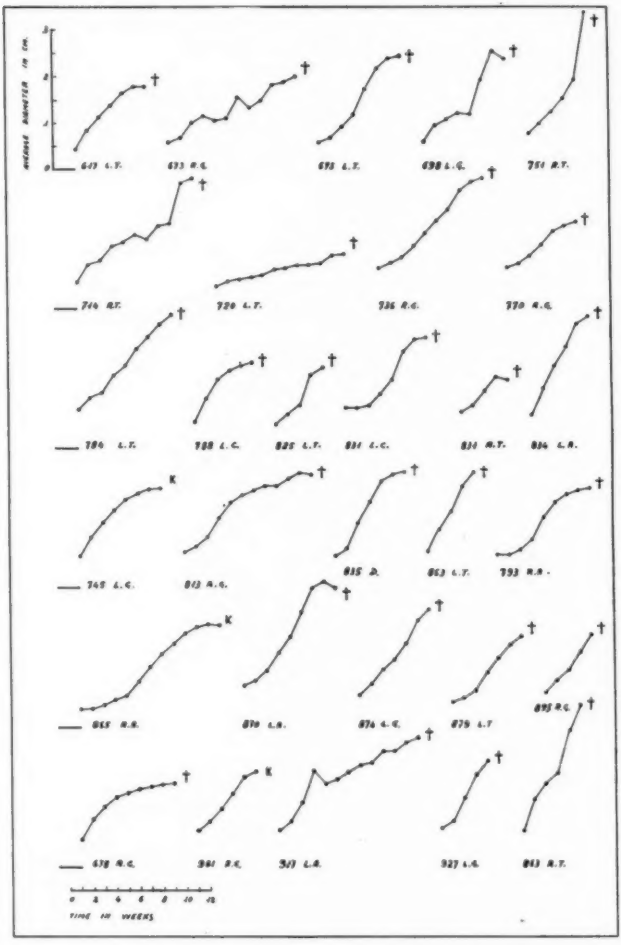
TABLE 3.—Types of mammary-tumor growth in breeding female mice of the C3H, BALB/c_f, and RIII strains

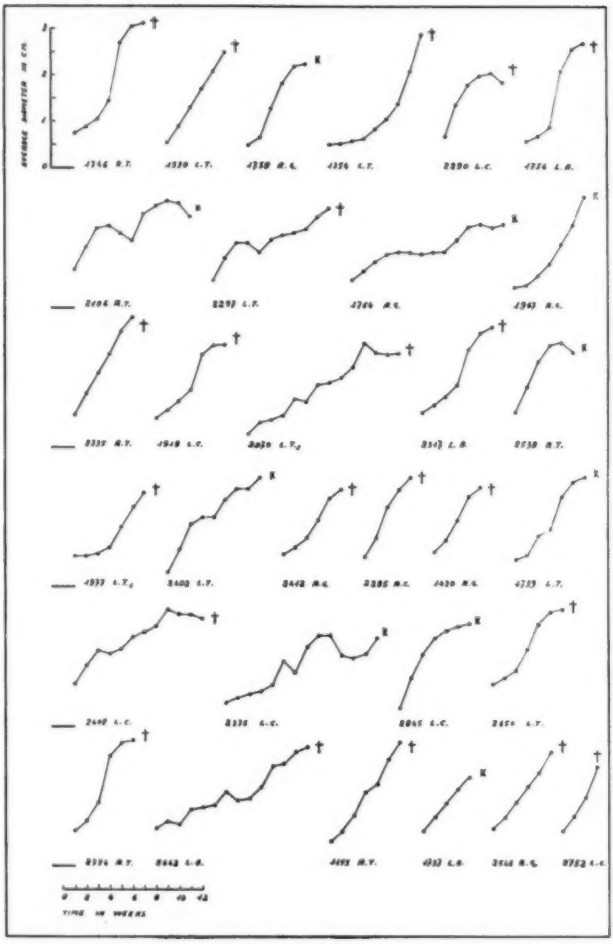
Strain	Number of palpable mammary tumors	Tumors discarded*	Tumors divided according to type of growth												
			Regular types of growth										Irregular growths		
			Sigmoidal		Linear		Parabolic		Exponential		Total				
			Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Chi square
C3H	76	20	22	39. 3	6	10. 7	7	12. 5	1	1. 8	36	64. 3	20	35. 7	—
BALB/c_f	254	72	41	22. 5	42	23. 1	20	11. 0	9	4. 9	112	61. 5	70	38. 5	0. 137†
RIII	75	15	4	6. 7	9	15. 0	4	6. 7	1	1. 6	18	30. 0	42	70. 0	13. 68‡
Total	405	107	67	22. 5	57	19. 1	31	10. 4	11	3. 7	166	55. 7	132	44. 3	

*All tumors measured less than 4 times and some tumors measured 4 or 5 times in which the type of growth was not recognizable.
†$0.8 > P > 0.7$.
‡$P < 0.001$.



TEXT-FIGURE 1.—Growth curves of 30 mammary tumors developed in 28 breeding female mice of strain C3H. Under each curve, number of animal and site of tumor growth are indicated. L. = left; R. = right; C. = cervical; T. = thoracic; A. = abdominal; G. = groin; D. = dorsal; † = dead spontaneously; K. = killed.

high-cancer-strain mice. This postulation agrees with a previous observation (*24*). However, it does not apply to the irregular tumors, the growth of which appears unpredictable at any time, perhaps because of their partial dependence on some extrinsic growth-stimulating factors, and/or their low degree of malignancy. This leads to the conclusion that the sigmoidal shape of growth may be observed only when some attributes, such as dedifferentiation, homogeny, and independence from extrinsic stimuli, have been gained by the tumor-cell populations.



TEXT-FIGURE 2.—Growth curves of 31 mammary tumors developed in 31 breeding female mice of strain BALB/c_t. Under each curve, number of animal and site of tumor growth are indicated. For abbreviations *see* text-figure 1.

DISCUSSION

The features of mammary tumorigenesis and the characteristics of tumor growth in female mice of the C3H, BALB/c_t, and RIII strains have been studied. These strains are susceptible to the development of mammary tumors and bear the milk agent. Hormonal stimulation of the breasts was provided by normal breeding. The results have pointed out that under the same conditions the three strains behave differently. Differences involve tumor incidence and age, number of tumors per mouse,

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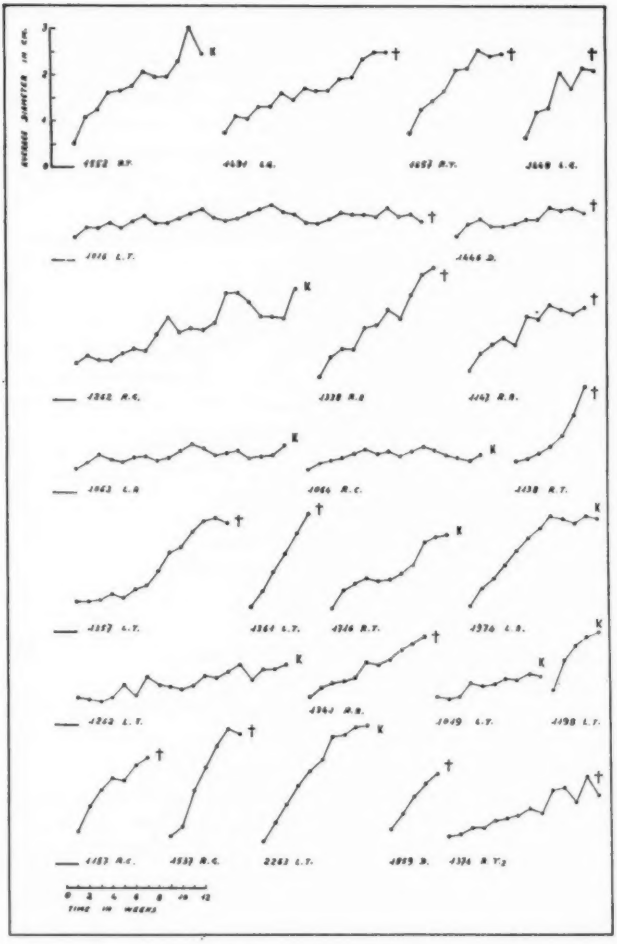

TEXT-FIGURE 3.—Growth curves of 25 mammary tumors developed in 24 breeding female mice of strain RIII. Under each curve, number of animal and site of tumor growth are indicated. For abbreviations *see* text-figure 1.

survival of tumor-bearing mice, and rate and type of tumor growth. However, the differences are slight between C3H and BALB/c_t mice, which possess a nearly parallel behavior, whereas in the RIII mice the behavior of tumors is peculiar enough to make this strain not comparable with the others.

The incidence, age at onset, and number of tumors per mouse are known to vary widely in different strains of mice in relation to differences in the causative factors. But there is no mention in the literature as to the occurrence of strain variations in the rate and type of tumor growth,

provided there is no extrinsic influence. This study has shown that most of the tumors occurring in C3H and BALB/c_t mice have a short duration, grow rapidly, rarely undergo partial regressions, and prefer regular types of growth, mainly a sigmoidal curve. By contrast, most tumors occurring in RIII strain have a long duration, grow slowly, frequently undergo partial regressions, and prefer irregular and unpredictable types of growth. This leads to the conclusion that even the characteristics of tumor growth are liable to variations in respect to the strain of host mice.

The growth curves of tumors in the RIII strain may suggest that some are benign with minimal growth rates and others are malignant. However, in spite of their slow and irresolute type of growth, the general tendency of mammary neoplasias occurring in RIII mice is toward progression to stages of increased autonomy (*25*, *26*), though it may require longer than the lifetime of the host (*15*). Moreover, the frequency of lung metastases for tumors of the breast in this strain is not lower than that shown by the other tumor strains investigated here (*27*, *28*). Therefore, the main difference between neoplasms of the C3H, BALB/c_t, and RIII strains lies on the level of autonomy reached by the tumor-cell populations at the time of their clinical detection and on the speed of progression, since a complete autonomy constitutes the common endpoint of all the breast tumors in high-cancer-strain mice.

The causes for the difference in tumor behavior in various strains are unknown. They might be only genetic, but it is interesting to note that in C3H and BALB/c_t strains, though their genetic origin is different, the behavior of tumors is parallel. The diet and other environmental factors which are known to influence the rate of tumor growth (*29*) did not have any role in the present results, since all animals were fed with the same standard diet and kept in the same environment.

Mammary tumors in mice, when established, have long been considered as autonomous growths and, therefore, independent of their causative factors (*30*, *31*). However, this opinion is already being revised because of the hormone dependence of some mammary growths (*15*, *25*, *26*). The hormonal stimulation of the breasts was different in the three strains investigated, because of their different fertility rates. Moreover, differences exist in the origins of the respective milk agents. C3H and BALB/c_t mice, which grow tumors with similar behavior, carry the same milk agent, whereas another type of agent is responsible for tumors in RIII mice. These differences in the type or efficiency of the causative factors might perhaps explain the other differences found in the characteristics of tumor growth among the strains investigated.

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Tumorigenesis in Transplanted Irradiated and Nonirradiated Ovaries [1, 2]

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SUMMARY

Over 300 hybrid mice (A × C57) were used to determine the incidences of ovarian tumors after irradiation and transplantation of ovaries. Female mice were given 87 or 175 r total-body radiation. Ovaries of these and nonirradiated donors were permitted to remain in place or transplanted subcutaneously or intratesticularly in male hosts. Some irradiated female mice received subcutaneous transplants of nonirradiated ovaries or of testes. Most of the intact and unilaterally castrated males had ovarian granulosa-cell tumors 600 to 700 days after a nonirradiated ovary was placed in the testis. Only about a third of the irradiated ovaries similarly grafted became tumorous. Nonirradiated ovaries grafted subcutaneously became tumorous in all the castrated male hosts, and two thirds of the irradiated ovaries became tumorous when transplanted in castrated male hosts. Approximately two thirds of the intact irradiated females and one third of the unilaterally ovariectomized, irradiated mice had ovarian tumors at death. Only 8 percent of the irradiated females that had an ovary transplanted subcutaneously from a young donor had ovarian tumors at death. Grafts of testis did not inhibit ovarian tumorigenesis. The environment provided by the male favored either ovarian tumorigenesis in grafted ovaries from both nonirradiated and irradiated donors or the growth of cells that had become tumorous. The incidence of lymphomas was higher in the male mice bearing grafts of irradiated ovaries. Two male mice bearing granulosa-cell tumors of the follicular type had mammary adenocarcinomas at death.—J. Nat. Cancer Inst. 26: 829–853, 1961.

OVARIAN TUMORS, usually of the granulosa-cell type, appeared frequently in mice subjected to procedures that presumably modified the intrinsic balance of hormones. Roentgen radiation adequate to destroy large numbers of ova and ovarian follicles resulted in the appearance of ovarian granulosa-cell tumors (*1*). Ovaries transplanted into castrated mice in areas draining venous blood through the liver also became tumorous (*2*). Similar tumors were observed originally in rats with intrasplenic ovarian grafts (*3*).

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Granulosa-cell tumors appeared infrequently in mice of most strains unless they were subjected to conditions that presumably would modify the internal hormonal environment (*4*). Some ovaries became tumorous when transplanted subcutaneously (*5*) or when removed and reimplanted into the ovarian bursa (*6*). There was a high incidence of tumors when ovaries were transplanted into the testes (*7*, *8*). The neoplastic ovarian responses have been difficult to evaluate or compare quantitatively. The duration of exposure of ovaries to the abnormal environment, the age of the ovary at which the environment became abnormal or was modified, and the strain of the mice differed from experiment to experiment. Furthermore, the criteria used to designate the endpoint of experimentation differed; for example, some investigators used histologic change as the endpoint, others, progressive growth in the animal of origin and/or transplantability to isologous hosts, or combinations of all criteria.

The present experiments were designed to study the quantitative differences in ovarian tumorigenesis under several different conditions. The sources of hormones were intrinsic except for a few female mice that received a follicle-stimulating preparation. The intrinsic hormonal environments were modified by castration, irradiation, or gland transplantation.

MATERIALS AND METHODS

Over 300 hybrid mice obtained by mating male and female mice of the A and C57 strains were used. These strains were maintained by brother × sister matings as an independent colony for approximately 11 years (originally from the colony of Dr. L. C. Strong). Because the incidence of ovarian tumors was the same whether the maternal parent was from the A strain (A71: 69♀ and 91♂ mice followed for 300 days or more) or from the C57 strain (A72: 54♀ and 41♂ mice followed for 300 days or more), the groups with reciprocal parents will not be considered separately. Seven mammary tumors appeared in mice of the A71 group (mice with strain A mothers) and two in the reciprocal hybrids. Approximately 300 mice were used to determine susceptibility to mammary tumors, when the mice were given different doses of estrogen or permitted to breed but not suckle their young. All mice were raised in the laboratory and were maintained on Purina laboratory chow and water *ad libitum*. They were vaccinated when 40 to 60 days old (*9*).

Mice from the same litter were usually placed in 2 or more different groups at the time the experiments were set up. The number of mice in each of the 8 groups of males ranged from 13 to 26 mice, and 7 groups of females ranged in number from 11 to 31. Female mice about 3 months old were exposed to total-body radiation of either 87 or 175 r. Radiation constants were: 200 kv; 15 ma; 45 cm TSD; 0.5 mm Cu and 1 mm Al, delivering 94 r per minute. Six mice were usually irradiated at one time in perforated plastic boxes measuring 6 × 6 × ⅞ inches, the doses being determined in air in the boxes. Ovaries were removed 2 weeks to several

months later and transplanted either subcutaneously or intratesticularly into male mice. Other male mice received ovaries from nonirradiated mice of comparable ages.

The mice were examined 6 times weekly until tumors developed, until death, or until their general condition indicated impending death. Satisfactory observations were made on 255 animals that survived more than 300 days. Some tumors were measured periodically to ascertain their growth characteristics. At necropsy all tissues or organs were inspected. Abnormal tissues were fixed in Bouin's fluid or 10 percent formalin for subsequent histologic study. All ovaries and ovarian grafts were so studied except when the tissue had undergone too much postmortem autolysis. At necropsy many specimens were photographed in color. The tumors were all measured and subsequently classified according to histologic types that were described previously (*10*). Some animals were not included in the tabular data because the tissue could not be observed adequately.

OBSERVATIONS

The variables in the experiments reported here were greater than the 15 groups of mice and included: (*a*) differences in amount of radiation, (*b*) differences in the age of the hosts and of the donors' tissues at the time of irradiation, (*c*) differences in the postirradiation period at which the ovary was transplanted, and (*d*) differences in the host's age at transplantation. For this reason most data will be presented in tables rather than in exposition.

Grafts of Irradiated Ovaries in Male Mice

Ovarian tumors appeared in only 12 of the 47 (tables 1–3) male mice in which irradiated ovaries were transplanted into the testes or the subcutis. The tumors that appeared were usually small; only one enlarged the testis in which it appeared (table 2, mouse 5). Only 2 tumors that arose in ovaries transplanted subcutaneously attained large size. The tumors were predominantly either tubular adenomas or modified fine trabecular types (II, IIa, V, or Va; fig. 6). Five of the tumors appeared in 9 ovaries obtained from donors exposed to 87 r total-body radiation. These ovaries were all transplanted from 14 to 17 days after the hosts were irradiated. Two tumors appeared among the 5 mice bearing ovaries grafted 14 to 17 days after radiation with 175 r.

Ten of the 16 castrated male hosts had tumors that arose in the ovaries grafted in the subcutis (table 4). Most of these tumors were also tubular adenomas of types II or IIa, though 2 were classed as luteomas. Four ovaries transplanted within 3 weeks of irradiation became tumorous, whereas only half (6–12) of the ovaries transplanted approximately 10 weeks after irradiation became tumorous. The age of the ovary when it was transplanted did not modify the tendency to become tumorous, though the duration of the postirradiation period did. The irradiated

TABLE 1.—Summary of observations on grafts of irradiated ovaries in testes of intact mice of the A71 group (mice 1–4 received 87 r; all others 175 r)

Number of animal	Period after irradiation ovary grafted (days)	Age of ovary at graft (days)	Age of host at graft (days)	Age at death (days)	Type and size* of tumor	Other tumors†
1	16	101	101	672	II/3	RCS
2	15	99	91	582	—	L
3	15	104	91	720	V/3	RCS, LA
4	15	89	105	666		
5	16	76	33	638		L
6	94	58	155	869	Va/3	L
7	71	53	130	862		L, LA
8	48	71	118	895		
9	58	72	120	849	—	RCS, LA
10	71	103	78	508		
11	66	62	86	490		RCS
12	66	62	71	464		RCS
13	76	93	74	744		LA, H
14	17	73	72	615	Va/1	L
15	17	73	72	476		

*Roman numeral refers to type of tumor (*see 10*). Denominator refers to size, *i.e.*, II/3 is a tubular adenoma about ⅓ the size of the normal testis.
†RCS = reticulum-cell sarcoma; L = lymphoid leukemia; LA = lung tumor; H = hepatoma.

TABLE 2.—Summary of observations on grafts of irradiated ovaries placed in testes of unilaterally castrated mice of the A71 groups (mice 1–5 received 87.5 r; others 175 r)

Number of animal	Period after irradiation ovary grafted (days)	Age of ovary at graft (days)	Age of host at graft (days)	Age at death (days)	Type and size* of tumor	Other tumors†
1	16	101	118	771	V/2	RCS
2	16	101	102	808	II/1	RCS
3	14	105	98	761	—	LA, L
4	14	105	97	732	—	
5	17	106	99	874	IIB/1+	
6	17	106	41	864		RCS
7	71	118	124	874	IIA/2	
8	71	53	131	750		RCS, LA
9	71	119	131	928		RCS
10	71	119	118	910		RCS
11	71	119	120	570		L
12	73	146	63	729	Va/2	L
13	73	146	63	729		RCS, LA

*Roman numeral refers to type of tumor. Denominator refers to size, *i.e.*, II/1 is a tubular adenoma that replaces entire testis.
†RCS = reticulum-cell sarcoma; LA = lung tumor; L = lymphoid leukemia.

ovaries more frequently became tumorous when transplanted into castrated males, though few tumors attained large size.

The average ages of the mice of each of the several groups and of the hosts of the nontumorous grafts were approximately 700 days (table 5).

TABLE 3.—Summary of observations on grafts of irradiated ovaries placed subcutaneously in intact male mice of the A7 group

Number of animal	Period after irradiation ovary grafted (days)	Age of ovary at graft (days)	Age of host at graft (days)	Age at death (days)	Type and size* of tumor	Other tumors†
1	15	118	101	808	IIa/L	
2	94	152	152	866		L
3	70	123	123	763		RCS
4	70	124	124	739		
5	70	119	117	556		RCS
6	83	131	118	775	—	RCS
7	82	130	112	735	—	LA
8	90	216	72	846		
9	90	216	72	777		RCS
10	90	216	72	636		
11	83	174	93	729	—	L
12	83	174	78	714	—	
13	62	128	86	728		RCS
14	62	128	86	799	—	LA
15	90	156	129	751	II/S	LA, L
16	73	146	63	667	V/L	LA
17	43	107	63	617		RCS
18	93	169	74	794		H
19	93	169	74	822		RCS

*Roman numeral refers to type of tumor. Denominator refers to size, *i.e.*, IIa/L is a tubular adenoma, large, two times or more.
†RCS = reticulum-cell sarcoma; L = lymphoid leukemia; LA = lung tumor; H = hepatoma.

TABLE 4.—Summary of observations on grafts of irradiated ovaries in subcutaneous tissues of castrated male mice of the A7 group

Number of animal	Age of ovary at graft (days)	Days post irradiation	Age of host at graft (days)	Age at death (days)	Type and size* of tumors	Other tumors†
1	101	14	91	662	IIa/3	RCS
2	90	17	41	859	IIa, IV/2	LA
3	105	17	41	864	Lut/2	PA
4	146	14	155	649	IV/3	LA
5	118	71	131	770		L, LA
6	118	71	131	928	IIa/2	L
7	113	72	118	849		RCS
8	113	72	120	855	IIa/3	LA
9	65	25	96	610	Lut/2	L
10	184	83	78	777		L
11	184	83	78	786		L
12	184	83	78	786	IIa/2	L
13	184	83	78	733		L
14	146	73	63	387		LA
15	146	73	63	617	III/4	
16	90	53	72	679	II/2	L

*Roman numerals designate the histologic type (*see 10*); Lut = luteoma. Figures given as the denominators refer to size of tumor: 1 means a tumor 3 mm or less in diameter; 2, 3 to 6 mm; 3, 6 to 9 mm; and 4, 10 mm or more.
†LA = lung adenoma; PA = pituitary adenoma; L = lymphoma; RCS = reticulum-cell sarcoma.

Because the ages of hosts at the time of transplantation averaged about 100 days, the grafts survived approximately 600 days. Within the limits of these experiments the age of the hosts at the time of transplantation (41–155 days) did not influence the response of the ovaries.

The nontumorous grafts were usually about 2 mm in diameter, and consisted of small areas of clear or ceroid-containing brownish cells, partially surrounded by bursa-like spaces lined by germinal epithelium and invaded to a variable extent by tubular or cleftlike epithelial spaces often demonstrably continuous with the germinal epithelium. A few grafts were large and cystic; fluid apparently accumulated in the clefts or bursa-like clefts that often partially surrounded the grafts. Those graft areas not demarcated from the adjacent tissues of the host by the germinal epithelial bursa usually blended with the host tissues. Sometimes macrophages and brownish cells apparently extended beyond the grafts into adjacent tissues.

TABLE 5.—Summary of observations on ovarian tumors in irradiated and nonirradiated ovaries transplanted into testes and subcutaneous tissue of male mice of the A71 group

Group and sex	Average age at grafting (days)	Number of animals	With ovarian tumors		Without ovarian tumors	
			Average age at death (days)	Number	Average age at death (days)	Number
♂♂ Irradiated ovary in testis	93	15	719	4	652	11
♂♂ Nonirradiated ovary in testis	100	15	738	13	596	2
♂♂ Irradiated ovary in testis	100	13	811	5	780	8
♂♂ Nonirradiated ovary in testis	73	26	747	20	755	6
♂♂ Irradiated ovaries subcutaneously	95	19	742	3	743	16
♂♂ Nonirradiated ovaries subcutaneously	101	15	753	3	673	12
♂♂ Irradiated ovaries subcutaneously	90	16	751	10	715	6
♂♂ Nonirradiated ovaries subcutaneously	92	13	692	13	—	—

Ovarian Tumors in Irradiated Female Mice

Eight of the 11 irradiated female mice had ovarian tumors; 6 were of histologic types IIa or Va. In addition, one mouse had 2 ovarian masses measuring 9 × 9 mm and 7 × 4 mm that contained so little viable tissue that they were not classified (table 6). The youngest mouse with an ovarian tumor was 386 days old and the oldest, 900. The tumors in these 2 mice were the same size and type. The average period of survival of the mice with and without ovarian tumors was 600 to 700 days after the time of irradiation (tables 6 and 7). The uteri of all the mice except #8 were moderately to well developed.

TABLE 6.—Summary of observations on ovarian and other tumors in irradiated intact female mice (mouse 1 received 87.5 r; all others 175 r)

Number of mouse	Age irradiated (days)	Postirradiation period (days)	Type and size* of tumor	Other tumors†
1	89	542	(N/3)	
2	30	592	III/4	LA
3	30	750	IIa/2	LA
4	32	900	Va/2	
5	32	484	Va/1	L
6	45	868		L
7	45	663	Va/2	L
8	116	389		L
9	94	724	Lut/4	L
10	94	386	Va, IIa/2	L
11	63	530	Va/2	RCS, LA

*N = necrotic. Roman numerals designate histologic type (*see 10*). Figures given as denominators refer to size: 1 means 3 mm or less in diameter; 2, 3 to 6 mm; 3, 6 to 9 mm; and 4, 10 mm or more. Designation in parentheses is lesion not considered tumor.

†LA = lung adenoma; L = lymphoma or lymphatic leukemia; RCS = reticulum-cell sarcoma.

TABLE 7.—Summary of observations on ovarian tumors in intact ovaries of irradiated mice, some with subcutaneous grafts of nonirradiated ovaries and testes

Group	Total number	With tumors		Without tumors	
		Number	Average survival after X ray	Number	Average survival after X ray
♀ ♀ Irradiated	11	8	615	3	631
♀ ♀ Irradiated	31	12	623	19	660
♀ ♀ Irradiated and graft of testis subcutaneously	17	9	593	8	696
♀ ♀ Irradiated and graft of ovary subcutaneously	25	2	730	23	570
♀ ♀ Irradiated	14	—	—	14	530
♀ ♀ Nonirradiated	11	0	—		
♀ ♀ Nonirradiated	15	0	—		
Total	124				

Ovarian Tumors in Unilaterally Ovariectomized, Irradiated Female Mice

Ovarian tumors appeared in 12 of 31 irradiated mice from which one ovary had been removed within a few weeks to 2 months after irradiation. The incidence, if the number of ovaries were considered rather than mice, is about the same as for the intact females. The ages at which the tumors appeared were also about the same in both groups. Postmortem changes prevented histologic diagnosis of 2 larger tumors observed prior to death. They were included because they were tumors, as indicated by their clinical history and gross appearance. Nine mice had small ovaries that consisted almost entirely of tubular ingrowths from the germinal epithelium. Except for size they resembled the tubular

adenomas, but previous experience had revealed that many lesions of this type did not grow progressively during the animal's lifespan. One tumor (4, table 8) grew from 7 × 9 mm to 13 × 22 mm in 157 days and was transplanted into mouse 24, table 8, in which it grew to 10 × 11 mm in 154 days. This tumor continues to produce estrogen effects in hosts after 6 years (*11*). Five of the mice had received follicle-stimulating hormones (FSH) for some time (*7*). Four other mice were set aside to be specific controls to the mice of this group. The treatment with FSH did not modify the initiation of ovarian tumors in the irradiated mice (table 8).

Three of the 13 mice receiving the smaller amount of radiation and 9 of the 18 mice that received 175 r had ovarian tumors. The periods of survival of both groups were similar. Some ovaries consisted almost entirely of tubular or cleftlike ingrowths of germinal epithelium and, although

TABLE 8.—Summary of observations on ovarian tumors and other tumors in irradiated mice of the A7 group from which one ovary was removed (mice numbered 1–13 received 87 r; all others 175 r)

Number of mouse	Age irradiated (days)	Age hemi-ovariectomized (days)	Postirradiation period (days)	Type and size* of tumors	Other tumors†
1	103	118	680	II/3	LA
2	86	101	643	II/2	L
3	86	101	613		
4	86	101	624	III/4	RCS
5‡	76	92	687		L
6§	76	92	717		LA, MCA
7‡	90	105	804	(II/1)	LA
8§	90	104	701	(II/1)	LA
9‡	90	104	804	(II/1)	RCS, LA
10§	90	104	694		L, MCA
11	90	106	643	(II/1)	RCS
12	90	106	777	(II/1)	AD
13	73	90	615		
14	59	152	648	?/4	
15	59	152	660	Va/4	
16	59	152	800	(V/1)	AD, L
17	59	152	434	?/4	
18	32	126	714		L
19	32	126	483		L
20	53	123	704	V/3	RCS, LA
21	53	123	512	II/2	RCS
22	47	118	661	III/3	L, LA
23	47	118	423		L
24	47	118	553	III/2	
25	48	119	710	(IIa/2)	RCS
26	48	120	633		
27	48	119	557	(II/1)	L, 2 LA
28‡	41	113	816		L
29	41	113	372	(II/1)	
30‡	58	130	603	IIa/2	RCS, LA
31§	58	130	789	I/4	PH

*Roman numerals indicate histologic type. Figures given as denominators refer to size of tumor (*see* footnote under table 6). Designations in parentheses are lesions not considered tumors; ? = histologic diagnosis not obtained.

†LA = lung adenoma; L = lymphoma or lymphatic leukemia; RCS = reticulum-cell sarcoma; MCA = mammary adenocarcinoma; AD = adrenal-cortical tumor; PH = pheochromocytoma.

‡Had received follicle-stimulating hormone (FSH).

§Controls to mice given FSH.

listed as type II, were not considered tumors because of their small size and less anaplastic appearance. The irradiated ovaries that were not involved in granulosa-cell tumors or luteomas were always small unless involved in lymphoma and resembled histologically the grafted ovaries in the males described.

Ovarian Tumors in Irradiated Female Mice Bearing Grafts of Testes and Ovaries

Nine of 17 irradiated female mice that had a testis from an 11- to 13-day-old donor transplanted subcutaneously had ovarian tumors at death (table 9). The size and types of tumors and ages of mice were similar to those of the irradiated females without testicular grafts. The testicular grafts were all small and contained predominantly seminiferous tubules lined by Sertoli's cells and a few spermatogonia. A few tubules contained scattered primary spermatocytes. The interstitial tissue was usually hyaline or fibrous when present in any significant amount. Glandular interstitial cells were not observed.

TABLE 9.—Summary of observations on ovarian and other tumors in irradiated intact female mice bearing subcutaneous graft of testis

Number of mouse	Age at X ray (days)	Age of host when testis transplanted (days)	Postirradiation period to death (days)	Size and type* of tumor	Other tumors†
1	42	225	694	(IIa/1)	LA, H, L
2	40	223	784	(II/1)	
3	40	223	810	IIa/2	L, LA
4	30	213	575	IIa/4	
5	30	213	556		H
6	30	213	633	III/3	
7	32	187	771	II/4	LA, L
8	32	187	436		LA
9	45	200	532		
10	45	200	731	IIa/2	RCS
11	94	121	603		RCS
12	105	108	748	Va/2	
13	103	104	553		
14	103	104	431	IIa/2	RCS
15	103	104	589		
16	116	117	758	{V/2 {IIa/2	
17	94	121	814	Va/2	RCS

*Roman numerals designate histologic type. Figures given as denominators refer to size of tumor (*see* footnote under table 6). Designations in parentheses are lesions not considered tumors.

†LA = lung adenoma; H = hepatoma; L = lymphoma or lymphatic leukemia; RCS = reticulum-cell sarcoma.

Only 2 of 25 mice that received after irradiation a subcutaneously placed graft of an ovary from an 11- to 26-day-old mouse had ovarian tumors at an average age of 766 or 730 days after irradiation (table 10). Twenty-three mice died without ovarian tumors after an average postirradiation survival period of 570 days. The incidence of tumors in the intact irradiated ovaries was lower than in mice of other groups (*cf.* tables 6 and 8 with

10). Two of the nonirradiated ovarian grafts in the subcutaneous tissues also became tumorous. One was a luteoma, one a small tumor of type Va. At the time of autopsy the other ovarian grafts were small and consisted largely of stroma and small cysts.

The transplants were made into hosts that ranged from approximately 60 to over 200 days of age and from several months to a few days after irradiation (table 10). The incidences of tumors were not modified significantly by the differences in scheduling of irradiation or transplantation. The mice with nonirradiated ovarian grafts had fewer tumors in their intact irradiated ovaries, whether the implants were made either 6 or 180 days after irradiation, than did the irradiated animals without ovarian grafts.

TABLE 10.—Summary of observations on ovarian and other tumors appearing in irradiated mice that subsequently received subcutaneously a graft of a nonirradiated ovary

Number of mouse	Age at X ray (days)	Age of host when ovary transplanted (days)	Postirradiation period to death (days)	Type and size of ovary graft	Type and size* of tumor ovary intact	Other tumors†
1	42	225	815	—	Va/1	RCS, LA, EC
2	40	223	436	—	—	F, RCS
3	30	213	646	Lut/2	IIa/2 IIa/2	L
4	30	213	407	—	—	L
5	32	187	479	—	—	L
6	32	187	484	—	—	RCS
7	63	218	815	—	—	L
8	63	218	712	—	—	L
9	105	108	524	—	—	
10	105	108	673	—	—	AD
11	94	121	503	—	—	
12	94	121	737	—	—	RCS
13	60	66	733	—	—	L
14	60	66	573	—	—	
15	63	69	322	—	—	
16	54	69	589	—	—	L
17	57	63	323	—	—	
18	57	63	705	—	—	RCS
19	65	71	550	—	—	MCA
20	54	69	678	—	—	L
21	52	58	550	—	—	
22	68	74	816	—	—	LA, L
23	54	69	320	—	—	
24	54	69	686	Va/1	—	2 LA
25	54	69	447	—	—	

*Figures given as denominators refer to size of tumor (*see* table 6).

†RCS = reticulum-cell sarcoma; LA = lung adenoma; EC = epidermoid carcinoma of the skin; F = fibrosarcoma; L = lymphoma or lymphatic leukemia; AD = adrenal-cortical tumor; MCA = mammary adenocarcinoma.

Grafts of Nonirradiated Ovaries in Male Mice

The incidence of tumors arising from nonirradiated grafts was high when the grafts were placed in the testes of the intact (13 of 15 mice) or hemicastrated (20 of 26) hosts and when placed subcutaneously in castrated hosts (13 of 13 mice; tables 11–14; figs. 1 and 2). The incidence

of tumors was particularly low when ovaries were transplanted subcutaneously in intact males (3 of 15 mice; table 14).

TABLE 11.—Summary of observations on grafts of nonirradiated ovaries in testes of intact male mice of the A7 group

Number of animal	Age of ovary at graft (days)	Age of host at graft (days)	Age at death (days)	Type and size* of tumor	Other tumors†
1	38	93	659	III/1	PA, RCS
2	38	93	631	IIa/2	L
3	46	132	760	Va/4	2 LA, RCS
4	39	73	828	IIa/L	LA, RCS
5	25	48	476		EC
6	307	136	626	/2	
7	125	70	762	Va/2	L
8	125	70	762	III/L	H, MCA, RCS
9	86	80	521	II/3	RCS
10	86	80	870	Va/3X	RCS
11	110	125	779	Va/2	RCS
12	110	125	716		
13	127	127	693	Va/1	RCS
14	127	129	821	Va/1	
15	127	129	883	Va/1	LA

*Roman numeral refers to type of tumor. Denominator refers to size (*see* footnote under table 1).
†PA = pituitary chromophobe adenoma; L = lymphoid leukemia; LA = lung tumor; RCS = reticulum-cell sarcoma; EC = epidermoid carcinoma of the skin; H = hepatoma; MCA = mammary adenocarcinoma.

TABLE 12.—Summary of observations on grafts of nonirradiated ovaries placed in testes of unilaterally castrated mice of the A72 group

Number of animal	Age of ovary at graft (days)	Age of host at graft (days)	Age at death (days)	Type and size* of tumor	Other tumors†
1	38	93	486	IIa/2	
2	40	131	762	?/2	RCS
3	39	35	664	Va/2	
4	26	48	945	Va/X	
5	119	98	727		2 LA
6	119	98	808	Lut/1	L
7	120	99	727	Va	RCS
8	120	63	668	V/2	
9	96	70	727	I/2X	
10	96	70	787	Va/2	RCS
11	101	65	787	IIa/1	LA
12	101	65	787	Va/1	LA
13	101	64	792	Va/1	
14	96	64	792	Va/2	
15	96	64	543	Va/2	RCS
16	96	64	792	Va/3X	
17	74	74	787	Va/1	LA
18	74	74	770	V/	L
19	70	70	679		L
20	70	70	787	V/1	
21	75	75	768		RCS
22	533	75	819		LA, RCS, PAP
23	533	75	819		PAP, RCS, LA
24	70	68	780	Va/2X	H
25	533	68	720		
26	70	68	781	Va/2X	RCS

*Roman numeral refers to type of tumor. Denominator refers to size (*see* footnote under table 1); ? = histologic diagnosis not obtained.
†RCS = reticulum-cell sarcoma; LA = lung tumor; L = lymphoid leukemia; PAP = papilloma of the skin; H = hepatoma.

TABLE 13.—Summary of observations on grafts of nonirradiated ovaries in subcutaneous tissues of castrated male mice of the A7 group

Number of animal	Age of ovary at graft (days)	Age of host at graft (days)	Age at death (days)	Type and size* of tumor	Other tumors†
1	131	40	974	IIa/3	LA
2	39	39	390	II,IIa/1	
3	36	35	648	IIa/2	RCS
4	81	307	571	II/2	RCS
5	136	307	742	IIa/3	PA, L
6	89	214	812	II,IIa/3	
7	70	70	676	II,III/2	MCA
8	70	70	725	IIa/2	
9	81	307	623	IIa/2	L
10	95	202	620	II,IIa/3	
11	110	116	875	IIa/3	LA, L
12	121	110	681	IIa/2	RCS
13	129	125	668	IIa/2	L

*Tumors were classified according to histologic types of predominating tissue. If two types were about equal, both were listed. Denominator refers to size: 1, less than 3 mm in diameter; 2, 3 to 6 mm; 3, 6 to 9 mm; 4, over 10 mm.

†L = leukemia or lymphoma; LA = lung adenoma; PA = pituitary adenoma; MCA = mammary adenocarcinoma; RCS = reticulum-cell sarcoma.

TABLE 14.—Summary of observations on subcutaneous grafts of nonirradiated ovaries in intact male mice of the A7 group

Number of animal	Age of ovary at graft (days)	Age of host at graft (days)	Age at death (days)	Type and size* of tumor	Other tumors†
1	58	58	845	IIa/S	
2	146	94	599		RCS
3	146	94	676		LA
4	216	79	623		LA
5	216	79	756		H
6	82	114	700	IIa/S	RCS
7	112	127	890		LA, RCS
8	112	127	790		RCS
9	112	127	680		ML
10	112	127	763		RCS
11	80	80	686		LA
12	80	80	795		RCS
13	80	80	414		RCS
14	24	97	401		
15	107	157	770	II/S	RCS

*Roman numeral refers to type of tumor. Denominator refers to size (*see* footnote under table 1).

†RCS = reticulum-cell sarcoma; LA = lung tumor; H = hepatoma; ML = myelogenous leukemia.

The tumors were histologically of the same types that arose from the irradiated ovaries. The tumor types II, IIa, V, and Va were most frequent among the intratesticular grafts and the relative frequencies of types V and Va were significantly higher than in other groups (figs. 3, 4, 5, and 7). At the time of necropsy the tumors were usually larger than those that arose from the irradiated ovaries. Whether or not this is of significance is problematic. Only three tumors enlarged the testis appreciably; others occupied variable portions of the testes. The adjacent seminiferous tubules were usually like those more remotely located and were comparable to those of mice of similar ages that had not carried ovarian grafts.

The nontumorous grafts were usually small and consisted of germinal epithelial ingrowths and a stroma of clear, lutein-like to ceroid-containing cells.

The subcutaneous grafts of the nonirradiated ovaries gave origin almost exclusively to tubular adenomas or modified tubular adenomas (tables 3 and 13). In the intact males 2 of the 3 tumors appearing in the graft of the irradiated ovaries were large and one was small (table 3), whereas the 3 granulosa tumors that appeared in mice with grafts of nonirradiated ovaries were small (table 13). The differences in periods of survival between the tumorous and nontumorous mice were not significant. Large granulosa-cell tumors were more frequently found in the castrated male mice bearing subcutaneous grafts of either irradiated (table 4) or nonirradiated (table 13) ovaries. Tumors appeared in all of the nonirradiated ovaries but in only about 60 percent of those that were irradiated.

Two of the ovarian tumors that arose in the intact ovaries of irradiated females were transplanted and grew. Both were tumors of type III and produced estrogen, as indicated by the responses of the host (*11*). Three tumors that arose in intratesticular grafts were also transplanted and grew in hosts of the same hybrid group (mice 3 and 24, table 12; mouse 8, table 11). One tumor that arose in an ovary was transplanted subcutaneously and also grew successfully (mouse 16, table 3).

Lymphomas and Reticulum-Cell Sarcomas

Of 63 male mice of the 4 groups bearing grafts of irradiated ovaries, 40 had either lymphomas or reticulum-cell sarcomas at death (tables 1–4). The lymphomas in 19 mice involved the mediastinal nodes and the thymus, but more frequently the intestinal nodules, mesenteric, celiac, and renal nodes, liver, and spleen were involved. Twenty of these tumors were reticulum-cell tumors, types A or B described by Dunn (*12*). The diagnoses were made histologically on group-cut test sections and are not incontrovertible. The different types of reticulum-cell lesions are not separated in the tabular data, and sometimes the tumors were mixed lymphomas and reticulum-cell tumors. In a few instances the ovarian grafts were also involved. The different lymphomas were variable in the extent of dissemination throughout the body and were composed, usually, of lymphoblasts of intermediate sizes.

Lymphomas appeared in 27 of 97 irradiated females that had one or both ovaries, and reticulum-cell sarcomas were present in 18 mice of this group. Seven lymphomas appeared in 26 nonirradiated females, 11 of which were gonadectomized. Of the 63 males that received irradiated ovaries as either subcutaneous or intratesticular grafts, 19 had lymphomas. In contrast, only 9 of 69 males that had nonirradiated ovaries transplanted similarly had lymphomas. The number of reticulum-cell sarcomas in these 2 groups was more nearly comparable (table 15). The high incidence of lymphomas and reticulum-cell sarcomas, 133 of 255 mice, was not unexpected, as these tumors occur frequently, especially the reticulum-cell tumor among old mice of these hybrid groups (table 16).

TABLE 15.—Incidence of lymphomas and reticulum-cell tumors in irradiated and nonirradiated hybrid mice (A × C57) bearing gonadal grafts and in certain control groups

	Group and treatment*	Number of mice	Number predominantly lymphomatous	Number of reticulum-cell sarcomas
1	♂♂ ♀ + t	15	5	5
2	♂♂ ♀ + t	13	3	7
3	♂♂ ♀ + sc	19	3	7
4	♂♂ ♀ + sc	16	8	2
		63	19	21
5	♀♀ +	11	6	1
6	♀♀ +	31	8	3
7	♀♀ + ♂ sc	17	3	4
8	♀♀ + ♀ sc	25	9	5
		84	26	13
9	♂♂ ♀ − t	15	2	8
10	♂♂ ♀ − t	26	3	8
11	♂♂ ♀ − sc	15	0	8
12	♂♂ ♀ − sc	13	4	3
		69	9	27
13	♀♀ +	13	1	5
14	♀♀ −	11	1	3
15	♀♀ −	15	6	2
		39	8	10

*♂♂ = intact male; ♂♂ = unilateral castrate; etc. Second designation of ♀ or ♂ refers to graft; + = an irradiated graft or animal; − = no irradiation of graft or animal; t = an ovarian graft in a testis; sc = an ovarian graft subcutaneously. Groups 1 to 12 are included in tables 1 to 4, 6, and 8 to 14; groups 13 to 15 acquired no ovarian tumor and are only listed.

TABLE 16.—Incidence of mammary and lymphoid tumors among estrogen-treated and control mice of hybrid groups (A × C57 and C57 × A)

Group	Sex	Treatment	Number of mice	Number of mammary cancers	Number of lymphomas
A♀ × C57♂	♂	Estrogen*	57	21	5 (254)†
"	♀	"	50	10	4 (310)
"	♂	—	16	—	6 (824)
"	♀	—	36	35 (300)	—
C57♀ × A♂	♂	Estrogen*	65	—	6 (341)
"	♀	"	33	—	1 (372)
"	♂	—	19	0	1 (795)
"	♂	—	31	7 (635)	9 (645)

*The estrogens used were estradiol benzoate, 25 and 16.6 μg weekly; estradiol dipropionate, 25 and 50 μg weekly; diethylstilbestrol, 250 μg weekly, and pellets of estrone weighing 2 to 3 mg. Treatment was started at about 30 days of age.

†Figures in parentheses represent average age of survival.

Pituitary Tumors

One mouse of group 14 (table 16) had a pituitary tumor measuring 4 × 5 mm at necropsy at 769 days. This tumor grew subsequent to transplantation and is being transplanted more than 4 years later, about 1

transfer generation per year. Histologically the tumor was composed of nongranular or chromophobe cells. A small chromophobe adenoma appeared in a castrated male (5, table 13).

Mammary Tumors

Two male mice had mammary adenocarcinomas at necropsy (tables 11 and 13). Both had granulosa-cell tumors of type III, a type usually associated with estrogen production, that had arisen in nonirradiated ovaries. It was impossible to determine whether the mammary glands of these mice were developed after the tumors appeared. The intact male with an intratesticular graft had an atrophic right testis and small seminal vesicles indicating high estrogen production (table 11). Mammary tumors appeared in 9 females; only 3 were more than 300 days old and hence were included in the tabular data (mice 6 and 10, table 8; mouse 19, table 10). Two mammary tumors appeared in mice with mothers from the C57 strain. The incidences of mammary tumors in estrogen-treated and forced-bred mice of the same hybrid groups were 29 and 97 percent when the mothers were from the A strain, and 0 and 23 percent when the mothers were from the C57 strain (table 16). The survival times rarely equaled 500 days among the estrogen-treated mice.

DISCUSSION

Ovaries that have been transplanted into the testes have retained ova for periods of a year or more (*13*) and produced enough estrogen to evoke mammary growth without completely impairing spermatogenesis. Ovaries transplanted subcutaneously in male mice of other strains apparently did not produce appreciable estrogen; at least the hosts showed little growth of the mammary rudiments (*5*).

Because the site of transplantation can modify the functional activity of a graft, one should probably not compare the hormonal effects of intratesticular and subcutaneous grafts. Ovarian grafts into the ears of mice, for example, produced androgenic effects (*14*) unless the mice were kept at temperatures higher than usual (*15*). Mice of different strains may also show different responses to ovarian grafts at different sites, though there is little evidence on this point. Only two male mice, both with nonirradiated ovarian grafts, had mammary adenocarcinomas (tables 11 and 13); one mouse had an intratesticular and one a subcutaneous graft. Both were in mice bearing tumors entirely or predominantly follicular (type III), which are usually associated with the production of estrogenic responses. Whether the pretumorous ovarian grafts or the hormonal activity of the tumors was responsible for the mammary growth and tumorigenesis could not be ascertained.

The impression was that the intragonadal environment might be more compatible with the maintenance of a more nearly normal hormonal func-

tion of the ovarian grafts. Corpora lutea were not observed in the ovarian grafts in male mice, though the stroma of the ovarian graft showed some lutein-like change. The male environment did not lead to corpus luteum formation and presumably would not be conducive to ovulation. The sex difference in the response of ovarian grafts was attributed to sex differences in function of the pituitary gland (*16*).

These observations indicate that the male produces relatively more follicle-stimulating hormone than the female and that the mechanisms presumably responsible for the regulation of the estrous cycles are not available to ovaries when grafted into males. The failure of ovarian tumor prevention in irradiated mice (*17, 18*) given amounts of testosterone adequate to avoid an appreciable increase in the levels of gonadotrophin subsequent to castration (*19*) adds further reservation concerning the requirement of augmented levels of gonadotrophin for ovarian tumorigenesis. The endocrine abnormality that is associated with ovarian tumorigenesis seems not so much one of level but of either continuity of end-organ exposure or the balance of pituitary hormones. The fact that only 2 of 25 mice with a subcutaneously transplanted nonirradiated ovary had tumors in the intact ovary certainly indicates that ovarian tissue, even when transplanted, reduces ovarian tumorigenesis (tables 5 and 7). Only 2 of the grafts (10%) in these females became tumorous in contrast to 20 percent in ovarian grafts in the intact male mice (table 14).

The probability that irradiation has a direct carcinogenic effect on the ovary is doubtful. Roentgen radiation in adequate amounts seems to impair the development of ova and associated hormone production, as was first noted by Brambell and Parkes (*20*). The modified hormone production in turn could account for the appearance of tumors. The toxic effects of 7,12-dimethylbenz[*a*]anthracene on ova in hybrid mice of the IF $\times$ C57 strains could account for the appearance of ovarian granulosa-cell tumors in mice given percutaneous application of this hydrocarbon (*21*). Mice so treated showed a gradual interruption of estrous cycles; however, the hybrid mice had many more ovarian tumors than mice of some other strains that were similarly treated. Furthermore, the injection of diethylstilbestrol or testosterone propionate did not prevent development of tumors in the IF mice given the hydrocarbon (*22*).

Partial removal of the ovaries of guinea pigs has resulted in prolonged periods of interrupted estrous cycles (*23*). The alterations of the cycles were attributed to the failure of the remaining ovarian tissue to re-establish an effective gonadal-hypophyseal balance. Transplanted ovaries rapidly lose most of their ova (*24*) and rarely, if ever, is the normal quota reattained. Transplanted ovaries usually become precociously anovular, or almost so, and thus incapable of provoking estrous cycles. It is possible that the ovarian tumors appearing in the transplanted ovaries are a response to the precocious aging of the tissue (*25, 26*). Mice with a hereditary early ovarian atrophy also frequently acquired ovarian tubular adenomas (*27*).

The appearance of ovarian granulosa-cell tumors in female mice with intrasplenic ovarian grafts and in parabiotic union with gonadectomized partners indicated that augmented levels of gonadotrophic hormones might be tumorigenic (*28*). The possibility that females so united might show a precocious loss of ova, however, must be considered as well as the probability of a more favorable endocrine environment. The prolonged treatment of mice with extrinsic gonadotrophins has not resulted in a significant increase in ovarian tumors (*7, 29*). The hormone production by ovaries was greatly modified in such mice; however, estrogen was predominant at first, then progesterone after 3 to 5 weeks, and androgen from 6 to 8 weeks later (*29*).

The difficulty of separating tumorigenesis from tumor growth must also be considered. When ovarian granulosa-cell tumors are transplanted, they frequently begin to grow more quickly in male than in female hosts (*11, 30, 31*). The primary neoplastic change may occur, but the environment favorable for the growth of the neoplasm may not be attainable until some subsequent time, for example, until they are in the presence of ovarian hormones or estrogens. The ovarian tumors are frequently hormone-dependent or partially so during the first few transplant generations. Even in male hosts the transplants may show appreciable growth only after several months. After growth begins it usually progresses quite rapidly, as if after a certain point the tumor could create its own autonomy. The ovaries in the irradiated mice (tables 7 and 8) may actually have been tumors for some time before becoming evident and merely awaiting an environment favorable for their growth, an environment that would have been attained had the ovaries been in males.

Transplantation itself may, however, provoke tumorigenesis. Ovaries transplanted intrasplenically for a short time and then reimplanted into the ovarian bursa became tumorous in some instances (*6*). The appearance of pituitary tumors in pituitary glands (*32*) transplanted subcutaneously in intact female hosts and of lymphomas and mammary tumors in transplanted lymph nodes and mammary tissue (*33*) suggests that transplantation must be given some consideration. The temporary ischemia coincident with transplantation may provoke neoplastic changes, or the death and resorption of many cells may have modified the surviving cells. Fischer (*33*) assumed that transplantation might permit a selection of a very few potentially malignant cells by inducing a great loss of normal cells that tend to restrict growth of the dormant malignant cells. The cellular debris, however, may favor growth of the potentially neoplastic cells until they can acquire autonomy. Furthermore, it is not impossible that the products of cell autolysis may become incorporated in normal cells to modify them irrevocably. Transplantation cannot be considered to be a passive experience. After successive generations of transplantation, tumors grow more rapidly and indicate that either a selection of cells or a transformation is taking place.

The material available provides inadequate critical data on hormone production by the tumors or by the gonadal transplants, even in the

gonadectomized animals, because nodular hyperplasia of the adrenal glands usually occurs. After transplantation into younger animals it is possible to determine more successfully the sources of estrogenic, progestational, or androgenic responses of the hosts (*11, 34*).

Most of the primary tumors that were followed grew very slowly; several were followed for 5 to 6 months after they first became palpable. Furthermore, the transplanted tumors during the first one or two transfer generations required 5 months to over a year to attain 10 mm or so in diameter.

The high incidence of lymphomas and reticulum-cell tumors is not unusual among mice of the C57 strain and their hybrids in this laboratory, especially when the mice are followed to old age. The tendency for a higher incidence of lymphomas among the males bearing irradiated grafts was noted, but its significance was questioned because of difficulties in diagnosis.

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PLATE 102

FIGURE 1.—Photograph showing testis and genital organs of unilaterally castrated male mouse (#11, table 12) that had nonirradiated ovary in testis for 722 days. Tumorous graft had replaced the normal testicular tissues. Tumor was a tubular adenoma showing some luteinization of intertubular tissue. Seminal vesicles were moderately enlarged. T = tumor, S = seminal vesicle; *arrow* indicates epididymis and mesorchium on the *right* side.

FIGURE 2.—Photomicrograph of section through left testis of mouse 15, table 12, 479 days after nonirradiated ovary had been transplanted into it. Finer structure was comparable to that shown in following figure.

FIGURE 3.—Photomicrograph of section of type Va ovarian tumor in testis of mouse that had received graft of nonirradiated ovary 629 days earlier (mouse 3, table 12). This tumor occupied over one half of testes. × 120

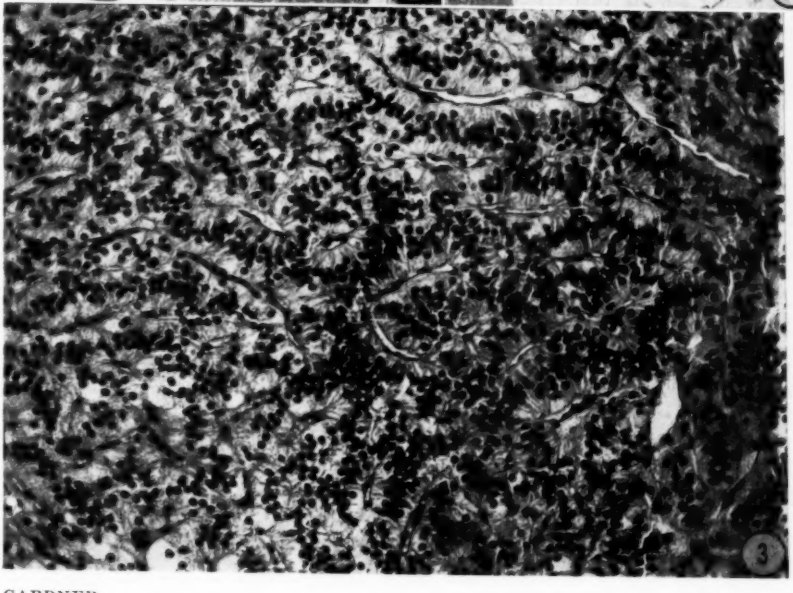
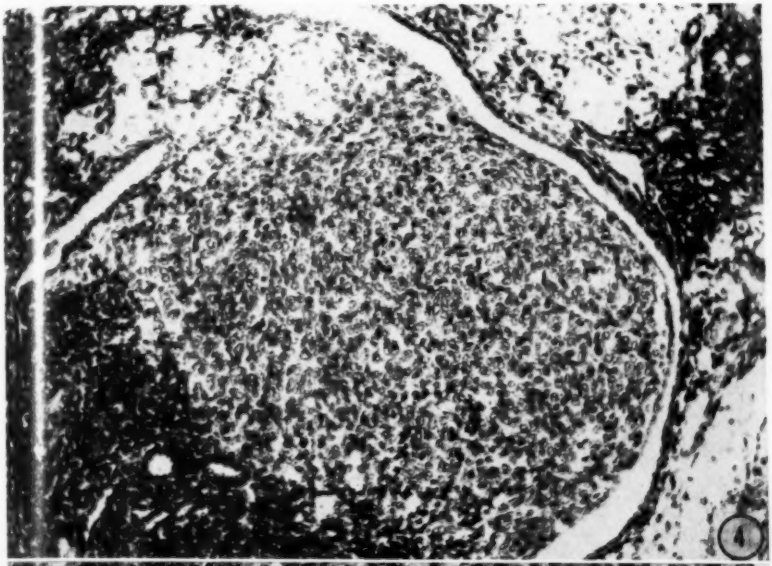


PLATE 103

FIGURE 4.—Photomicrograph of small section of tumor shown in figure 1. Although greater part of tumor was tubular adenoma, as shown at *lower left*, nodule of granulosa cells was present and partially surrounded by cleft lined by cells continuous with germinal epithelium. × 120

FIGURE 5.—Photomicrograph of section of tumor in ovarian graft in testis of mouse 15, table 12 (*see* fig. 2). This tumor was similar to that in figure 3, and shows transition of small cuboidal cells to larger cells. It seems probable that these tumors arise from tubular adenomas. × 120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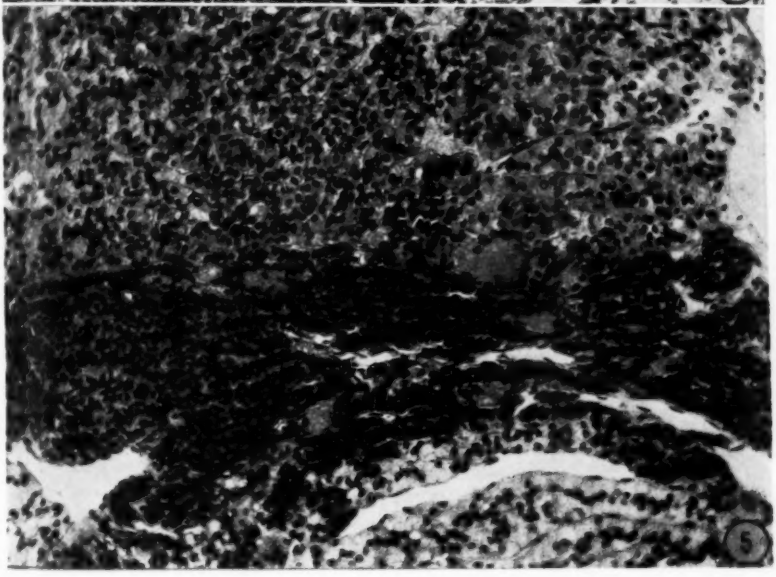
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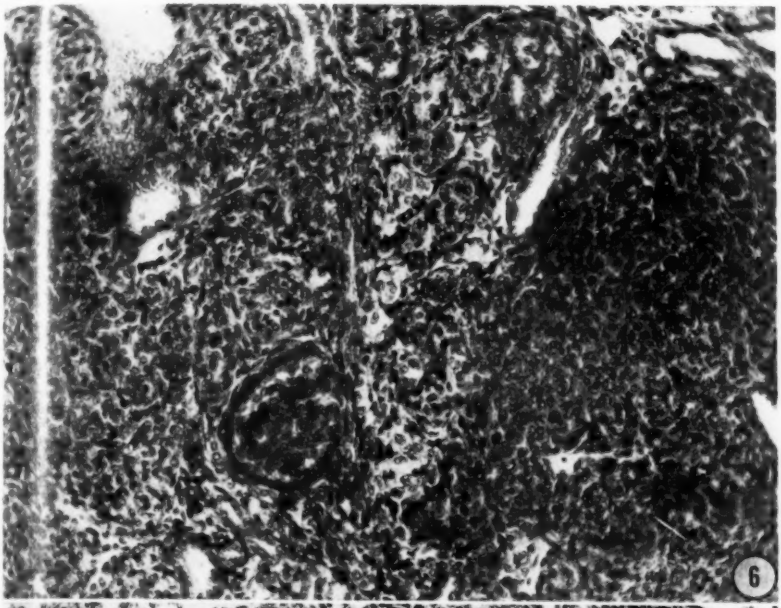
PLATE 104

FIGURE 6.—Photomicrograph of area of ovarian tumor that arose in mouse that had irradiated ovary implanted into testis for 750 days (#7, table 2). Although this tumor was classed as modified tubular adenoma (type IIa), this particular area had lost much of its tubular structure. This tumor was growing rapidly, as indicated by numerous mitotic figures. × 250

FIGURE 7.—Photomicrograph of area of tumor that arose in nonirradiated ovary that had been in testis for 755 days (#4, table 11). This tumor was type IIa. Another area of this tumor was luteomatous. Lutein cells in most of these tumors were not hyperplastic but appeared to differentiate from tubular and intertubular cells.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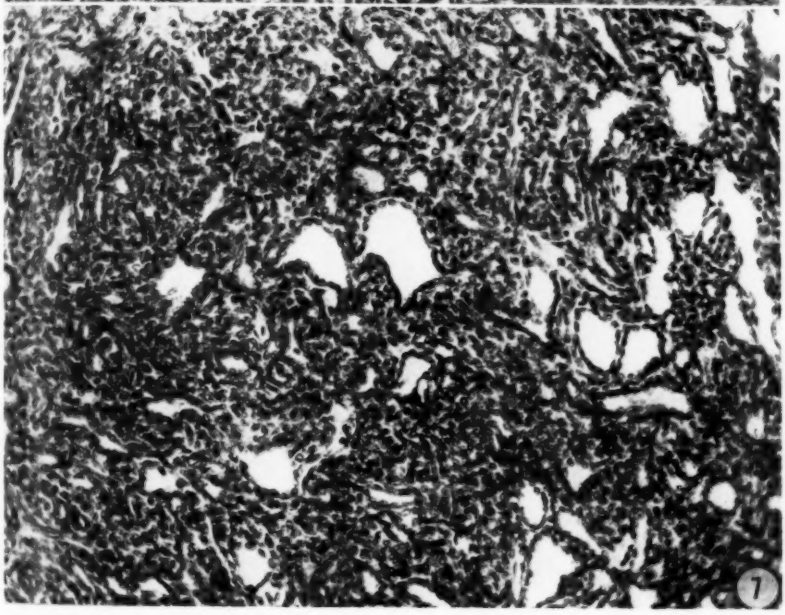
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Induction With Carbon Tetrachloride of Liver-Cell Carcinomas in Hamsters [1, 2, 3]

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SUMMARY

Carbon tetrachloride was administered by stomach tube to 10 female and 10 male Syrian golden hamsters, in weekly doses of 6.25 to 12.5 μl for 30 weeks. The livers of the animals dying during or after the period of treatment showed postnecrotic cirrhosis with regenerative hyperplastic nodules. All the animals, 5 females and 5 males, dying or killed 13 to 25 weeks after the end of the treatment, had 1 or more liver-cell carcinomas. No cholangiomatous tumors were observed. The induction, with *N*-2-fluorenylacetamide, of only cholangiomatous tumors and, with carbon tetrachloride, of only liver-cell tumors is discussed.—J. Nat. Cancer Inst. 26: 855–863, 1961.

THE PRESENT study is part of a larger investigation of the response of the Syrian golden hamster to carcinogens inducing neoplasms of the liver in other species. Some of these studies with hamsters, already reported, have recorded the induction of cholangiomatous lesions and cholangiocarcinomas with *N*-2-fluorenylacetamide (*1*) and negative results with *N*-dimethyl-*p*-phenylazoaniline, *N,N*-dimethyl-*p*-(*m*-tolylazo) aniline, thioacetamide, and ethionine (*2*). These observations, together with those of Miller and Miller and their collaborators (*3–5*), indicate that the response of the hamster to these chemicals is very different from that of the rat.

Carbon tetrachloride, administered orally over a long period, has been reported to induce hepatomas in several strains of mice (*6–9*), while similar studies in the rat yielded no liver tumors (*10–11*). The present report describes the induction, with carbon tetrachloride, of liver-cell carcinomas in the hamster.

MATERIAL AND METHODS

Ten female and 10 male Syrian golden hamsters (Abrams Small Stock Breeders, Chicago, Ill.), 12 weeks old, were used. They were housed in

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plastic cages on wood shavings in groups of 5, according to sex, and were given Rockland diet in pellets and tap water *ad libitum*. The treatment consisted of weekly administration by stomach tube of a 5 percent solution of CCl_4 (Fisher Scientific Co.) in corn oil (U.S.P.) for 30 weeks. During the first 7 weeks, 0.25 ml of the solution containing 12.5 μl of CCl_4 was given each week; this dose was then reduced to 0.125 ml and contained 6.25 μl of CCl_4. After the end of the treatment, the survivors were kept under observation for 25 additional weeks and then killed. A detailed pathological study of all animals was done, except for 1 female lost through cannibalism at the 28th week. The liver was fixed in 10 percent buffered formalin, Zenker-formalin solution, and absolute alcohol. Sections were stained with hematoxylin and eosin, Masson's trichrome, Snook's silver impregnation, periodic acid-Schiff with and without diastase, and Turnbull's blue method.

RESULTS

At the beginning of the experiment, the average weight of the animals was 109 g for the females and 99 g for the males; it varied irregularly during the 30 weeks of CCl_4 administration and was 104 g at the end of the treatment in both sexes. During the following period of observation, the weight increased and reached an average of 114 g in the females and 118 g in the males.

One female died at the 10th week of treatment; 3 females and 5 males died or were killed between the 17th and the 28th weeks. Three females died at the 41st, 43d, and 54th weeks, respectively; the surviving 3 females and 5 males were killed at the end of the 55th week.

The livers of all animals dying during the treatment and of the female that died at the 41st week showed changes consistent with postnecrotic cirrhosis. The livers were small and hard and had a nodular surface with irregular scarring involving all the lobes. Microscopic examination showed strands of fibrous tissue surrounding parenchymal nodules of varying size with obliteration of the normal lobular architecture (fig. 1). The regenerative hyperplastic nodules were composed of 2 to several cell-thick plates; the cells showed irregularities in the shape, size, and staining qualities of their cytoplasm and nucleus, with an uneven distribution of glycogen. Fatty metamorphosis was not prominent. In the animals dying within 48 hours after the treatment there were necrotic changes and hemorrhages involving the regenerated parenchyma. The fibrous septa were of irregular thickness and contained a moderate number of newly formed small bile ducts, inflammatory cells, and histiocytes laden with brown pigment positive to iron and periodic acid-Schiff reactions.

The other 10 animals killed or dying between the 43d and the 55th weeks had 1 or more liver-cell carcinomas with a total of 22 tumors: 12 in the 5 females and 10 in the 5 males. These tumors varied in size from

5 to 30 mm and were located in all the lobes: 7 in the left, 9 in the right, and 6 in the posterior lobes. Grossly, their grayish-pink color and firmer consistency differentiated them from the hyperplastic, regenerative nodules. Histologically, they were composed of atypical cells, often in mitosis, either in solid masses without any structure (fig. 2) or arranged in small nests and short trabeculae surrounded by dilated vascular spaces with numerous endothelial cells (fig. 3). The 2 patterns frequently coexisted in the same tumor. Reticulin fibers, demonstrated by silver impregnation, were found on many occasions completely surrounding small nests of tumor cells (fig. 4). This pattern was helpful in distinguishing liver-cell carcinomas from adenomatous nodules, the latter showing a more linear arrangement of the reticulin fibers. The tumors did not have a capsule, but compressed and invaded the surrounding parenchyma, which often showed necrotic changes (fig. 5). Areas of necrosis were seen also within the larger tumors. The liver tissue not involved by the tumors showed cirrhotic changes as in the animals dying during the treatment, but with less evident nodular hyperplasia. Well-circumscribed areas of hydropic degeneration were common (fig. 6). In addition, there were multiple, scattered, small foci of disorderly arranged atypical cells with large, hyperchromatic nuclei. These areas were not considered tumors, though it is recognized that they might have progressed to neoplasms. Bile-duct proliferation was inconspicuous, though a few conglomerates of microcysts and some dilated bile cysts were observed. One of the larger liver-cell carcinomas metastasized to the mesenteric and cervical lymph nodes. This tumor and 6 others were transplanted unsuccessfully. No other tumors or lesions of importance were seen in other organs.

DISCUSSION

It had become apparent from prior studies (*2*) that the hamster is quite resistant to the action of certain chemicals known to induce liver tumors in other species. A marked exception to this was *N*-2-fluorenylacetamide that gave rise to a high incidence of liver tumors in the hamster (*1*). This chemical, however, was found to induce only cholangiomatous tumors in this species, whereas in other test animals it gave rise to liver-cell tumors and, in addition, to many other tumors at different sites (*10*, *11*). An exception to this is an experiment reported by Maliugina (*12*), who found 2 hepatomas in 17 hamsters treated with *N*-2-fluorenylacetamide. In view of the fact that untreated hamsters, maintained on a stock diet, frequently developed cholangiomatous lesions (*1*), a relationship between this occurrence and the particular carcinogenic properties of *N*-2-fluorenylacetamide might be suspected. It is all the more interesting, therefore, that carbon tetrachloride gives rise to liver-cell carcinomas in the hamster, with a notable absence of cholangiomatous tumors. The susceptibility of the hamster to the carcinogenic action of

carbon tetrachloride is of additional interest in view of the fact that no reports are available in the literature on the induction, with the same chemical, of liver tumors in the rat. Even if these response differences among species might be overcome by modifications of diet and dosage, it is clear that the hamster differs markedly from the rat in its response to this group of carcinogenic chemicals.

Hepatomas have rarely been observed in hamsters (*2*, *12–15*). In control groups kept in this laboratory for a lifespan, a total of 145 female and 109 male hamsters, obtained from the same commercial breeder as the animals used in the present experiment and fed the same Rockland diet, did not develop liver-cell tumors (*1*, *16*). Moreover, no liver-cell tumors were observed in a group of 30 females and 50 males fed the same diet and given 0.5 ml of corn oil by stomach tube twice weekly for 45 weeks (*16*). It seems, therefore, that carbon tetrachloride has influenced the occurrence of a rare neoplasm in this species.

The diagnosis of malignancy in the liver tumors observed was based on histological criteria only, to which little was added by the solitary case of metastasis. The negative results of the transplantation study deserve further investigation. Many other tumors of hamsters have been successfully transplanted to noninbred hamsters in this and other laboratories. However, even in inbred mice the attempts to transplant carbon tetrachloride-induced hepatomas have not been homogeneously successful (*7*, *17–19*). Leduc and Wilson (*19*) suggested as an important factor the length of the interval between the last administration of carbon tetrachloride and the transplantation.

In many experiments with liver carcinogens in rats, it has been common to find both cholangiomas and hepatomas, and much discussion has been devoted to the histogenesis of these tumors (*20*). Although the present study does not exclude the possibility that bile-duct-cell tumors and liver-cell tumors might originate from a single type of cell or might transform one to another, these different lines of tumors may be induced in the hamsters by different agents.

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PLATES

PLATE 105

FIGURE 1.—Postnecrotic cirrhosis; regenerative nodules of various sizes surrounded by broad, fibrous strands. Male hamster, died during 20th week of treatment. Masson's trichrome. × 80

FIGURE 2.—Liver-cell carcinoma composed of atypical cells in solid arrangement, without organoid pattern. Male hamster killed at end of experiment. Hematoxylin and eosin. × 350

FIGURE 3.—Liver-cell carcinoma composed of cellular trabeculae separated by sinusoid vascular spaces; this tumor metastasized to mesenteric and cervical lymph nodes. Male hamster killed at end of experiment. Hematoxylin and eosin. × 200

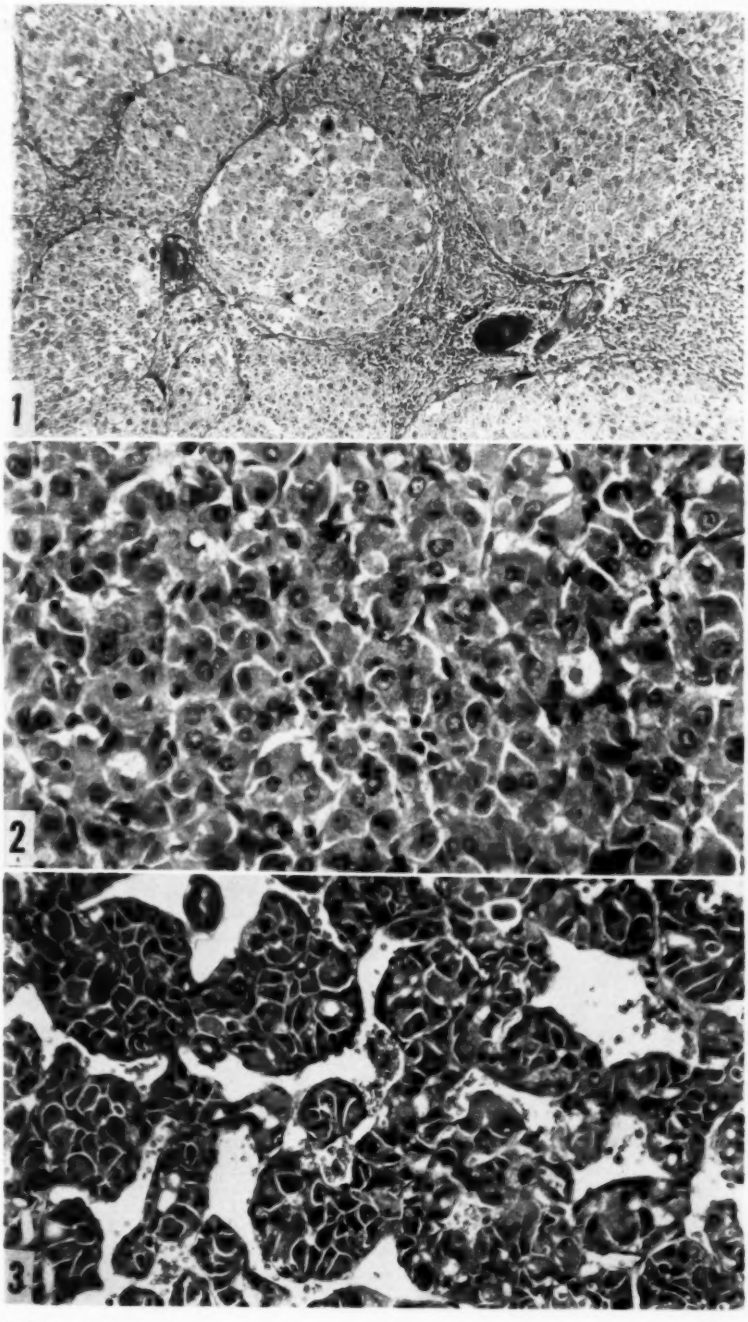
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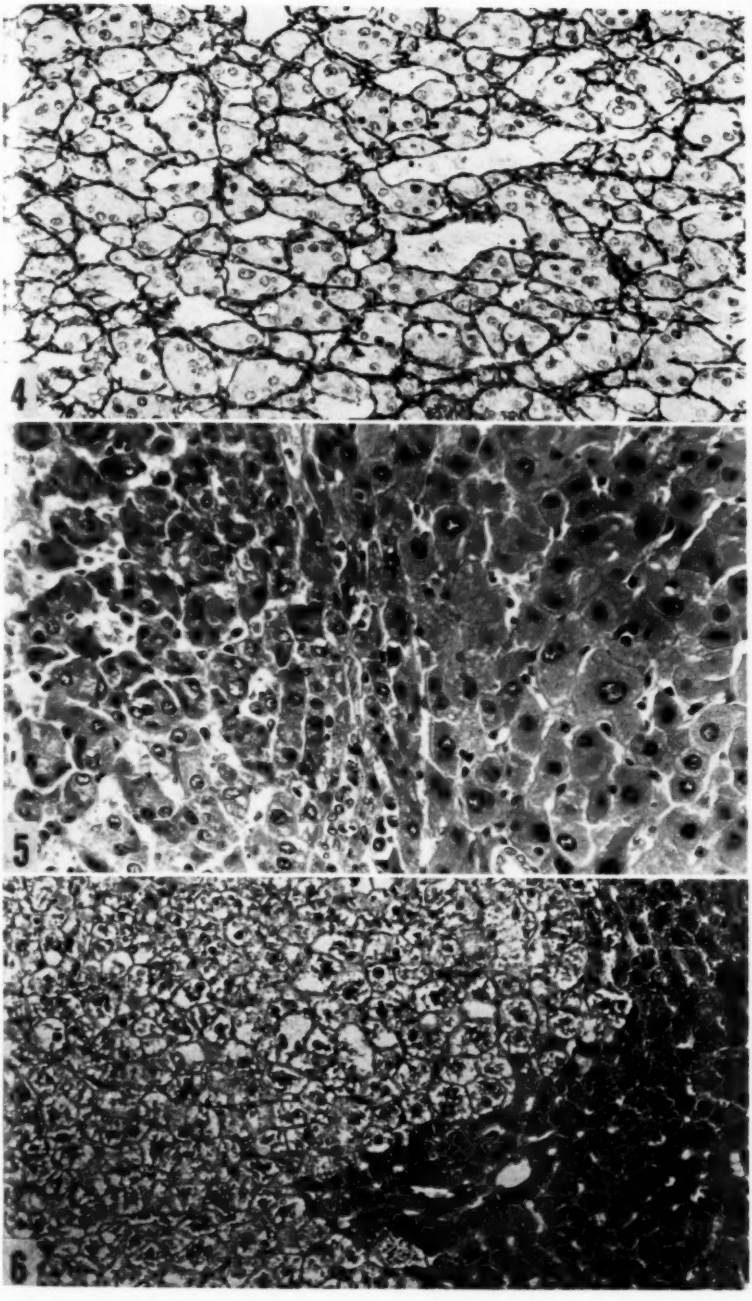
PLATE 106

FIGURE 4.—Liver-cell carcinoma, reticulum fibers completely surrounding small nests of cells. Female hamster killed at end of experiment. Snook's silver impregnation. × 180

FIGURE 5.—Liver-cell carcinoma compressing regenerated parenchyma. Female hamster killed at end of experiment. Hematoxylin and eosin. × 250

FIGURE 6.—Area of hydropic degeneration of parenchymal cells sharply demarcated against normal liver tissue. Female hamster killed at end of experiment. Hematoxylin and eosin. × 180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Modification of Characteristics of an *In Vitro* Mouse Cell Line After an Increase of Its Tumor-Producing Capacity [1, 2]

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SUMMARY

Three of nearly a hundred normal C3H mice, receiving subcutaneous injections of large inoculums of cells from the low-cancer NCTC 2555 clone (Sanford *et al.*), developed sarcomas at the inoculation site after long latent periods. Two cell strains established *in vitro* from these tumors were analyzed and compared with the parental clone and a constantly high-cancer NCTC 2472 clone. The two "derived" cell strains maintained a relatively high tumor-producing capacity during at least 8 to 9 months *in vitro*. The karyotype of each was like the parental line, especially in respect to biarmed chromosomes. The rate of cell multiplication remained unchanged, but morphological modifications, such as cytoplasmic ramifications and increase of mobility, were noted. No striking antigenic alterations of the "derived" cell lines could be evidenced by cross inoculations in mice. However, implantations on chick chorio-allantoic and chick mesonephros organ cultures allowed us to demonstrate a gradation of invasive capacity of different cell lines studied, parallel to their malignancy for isologous mice.—J. Nat. Cancer Inst. 26: 865–889, 1961.

FROM A clone derived *in vitro* from normal subcutaneous connective tissue of the C3H mouse, several cell lines have been obtained by Sanford *et al.* (*1*). These lines, after a prolonged period of culture *in vitro*, presented a wide range of tumor-producing capacities when inoculated into animals of the strain of origin.

A striking fact is that virtually all the sublines, including the "low-cancer" lines, occasionally produce proliferating tumors when inoculated into the animal in massive doses (*2*). These "low-cancer line" tumors can be transplanted serially in C3H mice. The new property of producing malignant growth in a high percentage of the inoculated animals becomes stabilized and seems thus to be linked with the transformed cells (*3*).

The purpose of the work reported here was to check the incidence of tumors originating from one of the low-cancer sublines maintained in our Institute and to analyze in several different systems possible new charac-

[1] Received for publication October 6, 1960.

[2] A short preliminary report of this work was presented at the Tenth Congress of Cell Biology in Paris, September, 1960.

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terstics appearing in connection with the increase in sarcoma-producing capacity of the derived tumor cells.

The general procedure was the following: Nearly 100 C3H mice received subcutaneous injections of large inoculums of cells from the clonal line NCTC 2555, which was characterized by a particularly low-cancer incidence (*4*) and failure to produce tumors in nonirradiated mice. In the study reported here, only 3 sarcomas appeared in the inoculated animals, 2 of which were explanted *in vitro* and adapted to continuous growth in culture. The explanted cells, designated further as "derived" cell lines, were characterized in the following ways during an 8- to 9-month period: cell morphology *in vitro* and *in vivo*, dynamic aspects of cell behavior (with the aid of phase-contrast microcinematography), rate of cell multiplication *in vitro*, rate of tumor growth in the animal, chromosome number and morphology, growth on the chorio-allantoic membrane of embryonated chick eggs, growth on chick-embryo organ cultures, immunological relationship, and reaction to certain virus infections.

During the course of these analyses, reference was made constantly to the low-cancer parental line, NCTC 2555, and, in addition, to another cell clone of the same group, NCTC 2472, a high-cancer line.

MATERIALS AND METHODS

Cultures of the high-cancer clone NCTC 2472 and of the low-cancer clone NCTC 2555 were received directly from Dr. K. K. Sanford, Tissue Culture Section, Laboratory of Biology, National Cancer Institute, Bethesda, Maryland. They will be referred to as cell lines N1 and N2, respectively. The cultures were maintained in our laboratory in a modified Morgan's 199 medium (*5*) with the amount of certain components increased as follows: glutamine 2, biotin and folic acid 5, pyridoxine and inositol 10, riboflavine, choline, and niacinamide 50, calcium pantothenate 150, and thiamine 250 times. The medium was completed with 15 percent normal, decomplemented horse serum.

Cultivated in Roux or milk-dilution bottles, cells were processed as mechanically dispersed, counted suspensions for the establishment of new cultures or for animal inoculations. To avoid any possibility of cross-cell contamination, the two lines were cultivated in different divisions of the laboratory and the media separately prepared.

To establish cell lines from mouse tumors, the tumor tissue was minced with scissors and dispersed with 0.25 percent trypsin in *p*H 7.5 phosphate-buffered salt solution without Ca and Mg ions. The cell suspension was cultivated in bottles directly on the glass surface in the medium.

For phase-contrast observation and microcinematography,[4] the living cultures were prepared on standard 12 × 32 mm coverslips in flattened tubes and transferred to special perfusion chambers adapted for direct

[4] The Kurt Michel microcinematography apparatus with a Zeiss phase-contrast microscope was used.

mounting of this size coverslips. Details of this technique have been described previously (*6*).

To check tumor-producing capacity, we inoculated subcutaneously a number of cells suspended in a standard 0.1 ml volume of medium into mice of the inbred C3H/He subline. Subcutaneous inoculation was preferable to intramuscular, since latent periods could be more accurately determined and the progress of tumor growth more easily followed. The tumors were measured by means of calipers twice weekly, and their average diameters (length + width ÷ 2) plotted against time to obtain growth curves.

For the chromosome studies, familiar cytological techniques were used. Cells were cultured on coverslips in flattened tubes for 1 or 2 days. Approximately 2 hours after the addition of 0.4 ml of a 0.0005 percent colchicine solution, the cultures were treated with hypotonic saline, then fixed and stained in aceto-orcein. Fifty metaphase cells of each of the 4 cell lines were characterized as to chromosome number and morphology.

Inoculation of cells onto the chorio-allantoic membrane of 10-day embryonated chick eggs was performed by the usual artificial air-chamber method. The detailed techniques for the inoculation of cells into the chick-embryo mesonephros organ cultures on agar substrate were described previously (*7*).

RESULTS

Origin of "Derived" Cell Lines

A total of 96 C3H mice were inoculated subcutaneously with 0.5 to 3×10^6 N2 cells. Only 3 of them presented palpable tumors at the inoculation site 70, 105, and 120 days later. Their growth was slow and localized, and the histological pictures showed in every case a well-vascularized sarcoma constituted by rather round, randomly arranged cells. Two of these tumors which appeared after a 70- and 105-day latency, designated N2T′ and $N2T^{bis}$, respectively, were explanted *in vitro* and showed first a mixed cell population composed essentially of: 1) cells with a clear cytoplasm and nuclei with very small and dispersed nucleoli, 2) cells with a more basophilic, denser cytoplasm and nuclei displaying large and intensely stained nucleoli. In passage, the second type of cell prevailed, and the cell population became quite homogeneous in both the N2T′ and $N2T^{bis}$ cell lines.

Rate of Multiplication *In Vitro*

During a period of 9 to 10 months of culture *in vitro*, these cell lines were transplanted every 7 to 10 days after 2 renewals of the medium. The increase in cell population during this period, evaluated by counting of viable (neutral-red-stained) cells, varied between 2 and 3 times in 7 days. When compared, the rates of cell multiplication in our standard

medium were the same for these derived cell lines as for the parental line N2. However, for the high-cancer N1 clone, during the same period and in the same medium, the average increase in cell number in 7 days was 3.2 times.

Cell Morphology

When the morphological appearance of the N2T′ and N2T^{bis} cells was compared with that of the N2 and of the reference high-cancer N1 line cells, the following observations were made:

The parental N2 line presented a rather uniform population of rounded cells, showing in dense cultures the tendency to form a pavement-like arrangement. Individual cells had usually a uniform, round, cytoplasmic area, surrounded by regularly moving, undulating membranes (figs. 2 and 5). Rare multinucleated giant cells were present, especially in old cultures. In the derived N2T′ and N2T^{bis} lines, the typical cell manifested rather extensive "tentacular" cytoplasmic ramifications (figs. 3 and 6). These ramifications were not passive retraction forms, but active cytoplasmic protrusions, which became evident from the analysis of microcinematography records. Normal rodlike mitochondria were regularly seen in these protrusions. The characteristic feature of these cells was their capacity to move and extend their ramifications over other cells, as can be seen in figure 6. Thus the dynamic behavior of the N2T lines approximated somewhat that of the high-cancer N1 clone (figs. 1 and 4).

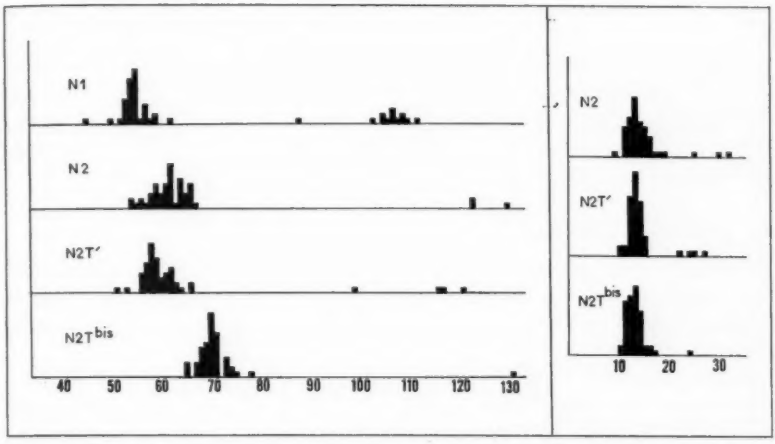
However, cells of the N2 type with regular, waving membranes could be seen as a persisting component of the cell population in the derived N2T′ and N2T^{bis} lines. Inversely, in the rather homogeneous cell population of the parental N2 clone, individual cells having a ramified cytoplasm and high mobility could be observed. Furthermore, transition from membranous to "tentacular" cytoplasm, as well as the reverse change, could be followed for individual cells.

No striking differences between the N2 and the derived N2T′ and N2T^{bis} lines were noted in nuclear size and total nucleolar mass as determined by apparent surface measurements. However, in N2T′ and N2T^{bis} cells, especially in earlier passages, there was a marked tendency toward a formation of one or two very large nucleoli, as compared with multiple nucleoli of lesser size in the N2 clone. The tendency to form one or two large nucleoli was likewise observed in cells of the high-cancer N1 clone.

Chromosomal Studies

The results of the chromosome analyses are illustrated by histograms in text-figures 1 and 2 (*see also* figs. 7 through 10). The modal chromosome number of the N2 parental line was 62, with counts ranging from 54 to 67, not including the few higher polypoid cells. This same line, as NCTC 2555, was examined by Chu *et al.* (*8*) and at that time had a modal number of 69. The modal number of the derived tumor line N2T′ was 58, with counts ranging from 51 to 66. The distribution of chromosome

counts in samples of these two populations was thus quite similar. However, the modal number of the N2T^{bis} line was 71, with a range of 65 to 78. There was only a slight overlap between this range of counts and those of the other lines.



TEXT-FIGURE 1.—Histograms of N1, N2, and "derived" N2T′ and N2T^{bis} lines. Distribution of chromosome numbers per 50 metaphases.

TEXT-FIGURE 2.—Distribution of biarmed chromosomes per 50 metaphases of parental N2 line and "derived" N2T′ and N2T^{bis} lines.

The derived tumor lines were inspected for the number and kind of biarmed chromosomes to determine whether they arose from the inoculums of the N2 clonal population. Chu *et al.* (*8*) found previously that the NCTC 2555 low-line clone was comprised of cells with a modal number of 11 biarmed chromosomes per cell, with a range of 8 to 14. In our laboratory, line N2 was represented by a modal number of 13 and a range of 9 to 19, as shown in text-figure 2. Similarly, the derived lines, N2T′ and N2T^{bis}, had modes of 13 and ranges of 10 to 15 and 10 to 17, respectively. Thus the increase in the total number of chromosomes of the N2T^{bis} line over that of the parental line was reflected as an increase of chromosomes with terminal centromeres rather than of the biarmed chromosomes atypical of normal mouse cells.

An attempt was made to classify the biarmed chromosomes of each of the 3 lines as to relative length and position of centromeres to detect possible differences between the lines in this respect (figs. 11 and 12). Three cells, each having 13 biarmed chromosomes, were selected from each line. An unusually long submedian chromosome, noted by Chu *et al.* (*8*), was found in most, but not all, of the cells examined. It can be seen that within any one cell line there was variation from one cell to another, with no clear-cut differences between the lines. In fact, the resemblance between the parental and derived lines was considerable.

The N1 line (NCTC 2472, high-line clone) had a modal chromosome number of 55, as was observed approximately 2 years earlier by Chu *et al.* At that time the line was noted to have 0 to 2 biarmed chromosomes per cell, with a modal number of 2. In our laboratory, the redesignated N1 line was without biarmed chromosomes in 76 percent of the cells examined, while the remainder contained 1 or, rarely, 2. One or two minute chromosomes were present in only 25 percent of the cells examined. In addition, a telocentric chromosome, markedly longer than the other chromosomes in these cells and longer than those seen in normal cells, was observed in at least 84 percent of the analyzed cells. Approximately one quarter of the N1 population consisted of higher polyploids, which were infrequent in the N2 lines.

Tumor-Producing Capacity of Derived Cell Lines

As previously mentioned, only the subcutaneous route of inoculation was used, and tumor growth was graphically represented according to tumor-diameter measurements. The latent period could be evaluated precisely in this way.

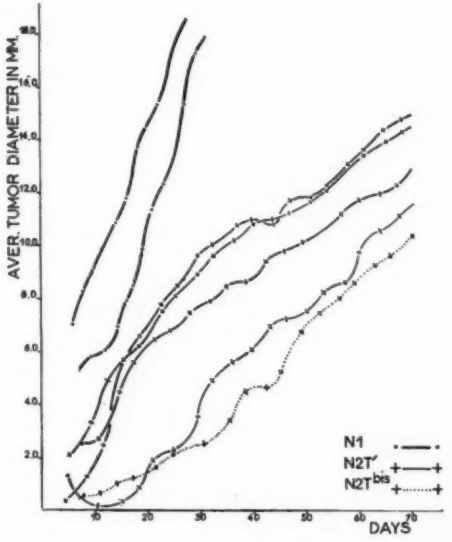
During a period of 6 months, a total of 77 mice, mostly 15 to 18 g males, were inoculated with 5×10^5 to 6×10^6 cells of the N2T′ line. Of these there were 67 (87%) takes, 86 percent of which showed progressive growth. With the $N2T^{bis}$ line, the takes were less frequent (51.5%) and more of them regressed (table 1).

TABLE 1.—Tabulation of tumor takes and progressive tumors for mice receiving 5×10^5 to 6×10^6 cells

Cell line	Inoculum size	Number of mice	Takes	
			Number	Percent
N1	2.5×10^6	14	13	92.9
	1×10^6	51	50	98.0
	5×10^5	30	27	90.0
	Totals	95	90	94.7
N2	5×10^6 to 3×10^6	92	3	2.7
N2T′	6×10^6	5	3	60.0
	2.5×10^6	36	35	97.2
	1×10^6	26	19	73.1
	5×10^5	10	10	100.0
	Totals	77	67	87.0
$N2T^{bis}$	$5–6 \times 10^6$	12	10	83.3
	2.5×10^6	21	7	33.3
	Totals	33	17	51.5

The growth rate, expressed as a curve of average tumor-diameter increase, was one of the most characteristic features of growth in the animal for each of the cancer-producing cell lines. Text-figure 3 compares 3 groups of curves of average growth of the N1, N2T′, and $N2T^{bis}$ lines in mice inoculated with 2.5×10^6 cells. The differences evidenced

by the curves were characteristic and observed repeatedly in several experiments through a period of many months.



TEXT-FIGURE 3.—Growth curves of tumors produced with N1, N2T′, and $N2T^{bis}$ cells obtained with a standard subcutaneous inoculation of 2.5×10^6 cells.

Tumor Characteristics

Tumors of the high-cancer clonal line N1 grew rapidly and reached a diameter of 30 mm or more in 6 to 8 weeks, killing the mice without showing, however, the tendency to invade the peritoneal cavity, thorax, or lymph nodes. Even at the final stage there was little necrosis in the center of the tumor mass and no ulceration.

In contrast, the slowly growing N2T′ and $N2T^{bis}$ tumors formed rather early necrotic and ulcerated centers, with bordering areas of viable tissue which progressed slowly but steadily. Mice could survive as long as 6 months with these tumors, which generally did not regress, once established. In mice bearing these slowly growing tumors of 5 to 6 months' duration, a considerable enlargement of the spleen and lymph nodes and metastatic tumor masses in axillary and inguinal lymph nodes, peritoneum, lungs, and kidney was observed.

Immunity Reactions

Sanford *et al.* (*2*) emphasized the existence of cross-immunity between different cell lines of this group. They also suggested that negative results obtained in mice after inoculation of low-cancer cell lines may result from an immune response of the inoculated animal rapid enough to pre-

vent or overcome the tumor growth. To check this under our conditions, the following experiments were performed: Mice remaining negative after injection of low-cancer N2 cells were reinoculated at a different site with 1×10^6 cells of the high-cancer N1 line, as were non-preinoculated controls.

In 12 mice preinoculated with 3×10^6 N2 cells 140 to 160 days before, the growth of N1 cells was comparable to that of the controls. However, in others which were preinoculated 99, 35, and 34 days before, some N1 tumors regressed completely, some were slowed down—then resumed progressive growth, and others underwent an eclipse period and reappeared after approximately 3 weeks.

In another experiment, 2.5×10^6 N2 cells were mixed in the same inoculum with 1×10^6 or 5×10^5 cells of the N1 line. Some transient depression of tumor growth between the 7th and the 15th day was observed, but the rate and level of subsequent tumor growth approximated that obtained from the inoculation of N1 alone.

To check the immunological relation between the N2 and the directly derived N2T′ and $N2T^{bis}$ lines, groups of 10 mice were inoculated with 2.5×10^6 N2, N2T′, or $N2T^{bis}$ cells. Parallel and similar groups of mice were inoculated with 2.5×10^6 N2 cells on the left side and with the same number of either N2T′ or $N2T^{bis}$ cells on the right side. In neither group did tumors appear on the left side, but the number of takes and the rate of growth were nearly the same in the doubly inoculated mice as in the controls.

To see whether in the N2T′ cells a new antigen was present which could induce a specific immune reaction in isologous mice in a more efficient way than the original N2 cells, the following experiment was performed: Uniform groups of 8 C3H males received subcutaneous injections of 3×10^6 cells of either the N2 or N2T′ strains, killed by repeated 3 times rapid freezing in an alcohol-dry-ice mixture and rapid thawing. They were challenged 19 days later with an inoculum of 1×10^6 N2T′ cells. This experiment was performed 9 months after the establishment of the N2T′ cell line, when the tumor-producing capacity of this strain had become lowered. Only 2 in 7 control mice developed tumors. Unexpectedly, all 6 surviving mice in the group preinoculated with the frozen N2T′ cells and 3 of 6 preinoculated with the frozen N2 cells developed progressively growing tumors. Thus, no inhibiting immunity reaction could be induced by an isoantigen from frozen N2T′ cells. However, on the basis of these results, an "enhancement" reaction of an immunologically specific character can be envisaged.

In other experiments, 25 million spleen cells from normal or tumor-bearing animals were admixed to 1 million N2T′ cells and inoculated subcutaneously in young C3H males. No inhibition of tumor growth was observed in mice receiving an admixture of spleen cells extracted from mice bearing large, well-developed 5-month N2T′ tumors (8 takes in 8) when compared with control mice inoculated with the N2T′ cells only (6 takes in 7). A control group of mice receiving the admixture of

normal spleen cells showed a significant inhibition of tumor growth (2 takes in 8 mice).

Grafts on Chorio-Allantoic Membrane and on Chick-Embryo Organ Cultures

Since the cancer-producing capacity of the cell lines under study could be interpreted as a result of a lessening, suppression, or overcoming of the immunity reaction of the host, experiments were undertaken in which growth of these cell lines was checked in biological systems known to be immunologically nonresponsive.

One of these systems was that of grafts on the chorio-allantoic membrane of embryonated chick eggs. With a standard inoculum of 1 million cells suspended in 0.1 ml of phosphate-buffered salt solution, the high-cancer N1 line regularly produced tumor-like nodules, which developed abundantly under the superficial ectodermal sheet and reached 6 to 8 mm in diameter in 8 days. The N2 cells, inoculated in the same way, did not grow at all. However, with both the derived N2T′ and N2T^{bis} lines, growth and formation of infiltrating nodules of 3 to 5 mm in diameter were observed in several experiments (figs. 13 through 15). In every case, the tumor-like nodules which developed on the chorio-allantoic membrane were well vascularized.

The other immunologically nonreactive system was that of cultures of embryonic kidneys of the chick (mesonephros) developing on agar sheets containing markedly reduced amounts of nutritive elements. As was formerly demonstrated by Wolff and Haffen (*7*), cultured cells placed directly on the agar degenerate rapidly, and the implanted cells can survive only if they grow into the embryonic organs.

The detailed results of these experiments are published separately (*9*). Briefly, dense suspensions of N1, N2, N2T′, and N2T^{bis} cells were deposited as microdrops on the surface of 3-day chick mesonephros organ cultures. After 6 to 7 days of symbiotic growth, some of these cultures were fixed and some transferred to other freshly prepared mesonephros organ cultures. The results of several experiments, which extended over a period of 6 months, were in agreement with the observations obtained with chorio-allantoic grafts: The N1 cells readily infiltrated the embryonic kidneys, showed a certain degree of proliferation inside the infiltrated mesonephros, and could undergo several passages. The N2 cells remained on the surface of the organ fragments and showed extensive degeneration and no infiltration whatsoever. However, the N2T′ and N2T^{bis} lines displayed a marked capacity to penetrate the chick mesonephros fragments and could even be occasionally transplanted, though less easily than the N1 cells.

Sensitivity to Viruses

No differences were noted between the N1, N2, N2T′, and N2T^{bis} cell lines in their reactions to infection with viruses of the EMC-Mengo encephalomyelitis group. The cells of all assayed lines were rapidly

destroyed when infected with a multiplicity of 1 to 5 infective virus units per cell, and in every case the titers of produced virus attained nearly the same levels.

However, definite difference appeared when the different cell lines were infected with the tumor-producing SE polyoma virus (*10*). More detailed data concerning these experiments will be published separately. It can be mentioned here that under comparable conditions of virus inoculation with 10^5 to 10^6 infecting doses per culture of nearly 3×10^5 cells, the N1 cells were entirely destroyed in less than 15 days, whereas in parallel N2 cell cultures, after partial destruction of the cultures, a certain number of cells survived. Latently infected but proliferating cultures could be obtained regularly in this way. Constant production of hemagglutinating and infectious virus material was observed in these cultures for a period of at least 7 months. The N2T′ and N2T^{bis} cells reacted in this respect in the same way as the parental N2 line.

DISCUSSION

To check new characters in a cell population after an abrupt increase of its tumor-producing capacity, we selected as a starting point in our experiments the NCTC clone 2555 (N2), which rarely produced tumors after inoculation in C3H mice.

We had no information to decide whether the striking increase of tumor-producing capacity after one successful passage through the animal was due to an inductive mechanism which operated on the inoculated cells or to a selection of pre-existent malignant cells that became a majority under the pressure of a changed environment. Yet, the comparative study of the parental and derived cell lines offered a good opportunity to check, in otherwise similar material, characteristics which might have appeared or become intensified in these cell populations simultaneously with the increased malignancy.

The N2T′ and N2T^{bis} cell lines obtained from two tumors produced with the low-cancer N2 line, despite their extremely long latent periods of 70 and 105 days, can be considered with high probability as direct descendents of the inoculated cells. This is concluded especially on the basis of the close similarity of the karyotype of all three lines: The mean chromosome numbers are found in a predominantly hypotetraploid area between the 56 and 73 values. Furthermore, the number of metacentric chromosomes are relatively high for all these lines between 8 and 15 with a common modal number of 13. These two features are characteristic of these cells and sufficiently unusual for mouse cells, either normal or cancerous, to make their origin from the inoculated host doubtful.

The data of Sanford *et al.* (*2*) were confirmed: In the derived cell lines obtained *in vitro* from the rare tumors produced with a low-cancer cell line, the tumor-producing capacity becomes stabililized on a nearly constant level during at least 7 to 8 months. Not only was the proportion of takes, which averaged 87 percent for the N2T′ line and 51 percent for

the N2T^{bis} line, characteristic and nearly constant for these cell lines during this period, but also were the latent period, rate of growth, and histological aspect of the produced tumors. So the extensive ulceration of the N2T′ tumors, together with hyperplasia of the lymphatic system and metastases in different organs observed in mice bearing old tumors, were peculiar to this subline.

These characteristics were not shared by the reference N1 (NCTC clone 2472), which has shown a constant high tumor-producing capacity for many years. In our hands, the N1 cells produced, when inoculated subcutaneously, spindle-cell sarcomas having a much shorter latent period, a rate of growth much faster than the N2T lines, and no tendency to give metastases or hyperplasia of the lymphoid system.

The following conclusion can be drawn from the comparison of cell characteristics directly observed *in vitro* for the N2 and the derived N2T′ and N2T^{bis} lines:

1) The rate of growth during a period of 8 months of culture *in vitro* was essentially the same for all of them.

2) The proportion of cells displaying extensive cytoplasmic ramifications, rapidly changing their shape and position without signs of contact inhibition in respect to other cells, appeared much higher in the derived than in the parental N2 cell line. These characteristics are reminiscent of certain aspects of the high cancer N1 cells, which also produce extensively moving cytoplasmic ramifications. However, despite these transformations, cells of the N2 family and the N1 cells never reach a state of morphological identity.

3) It must be emphasized that cells with ramified cytoplasm characteristic for the N2T lines were found occasionally in the parental N2 line. Similarly, cells of the N2 type, having regular membranes and firmly attached to the glass substrate occur in the derived N2T lines. Furthermore, transformation from one cell type to the other was observed. Even though the ramified or membranous forms in the N2 family must be considered a state rather than a rigid form, a tendency to appear in the ramified, "tentacular" state seems to be proper to the derived cancer-producing N2T cells.

4) Another morphological feature of the N2T lines, and one which brings them closer to the high-cancer N1 clone, is an increased proportion of nuclei with few but very large nucleoli.

5) When the chromosomal constitution is considered, it is apparent that no distinctive differences exist between the N2 line and the derived N2T′ and N2T^{bis} lines, with the single exception of a definite increase in chromosome number of the latter. The number and type of biarmed chromosomes, peculiar to the N2 cell family, are strikingly similar for all the lines.

Thus, the marked change in tumor-producing capacity of both the derived lines was not related to any consistent, apparent changes in chromosomal characteristics. Indeed, a more sustained relationship exists between this capacity and certain other cell characteristics, such as morphological and dynamic aspects of the cytoplasm and nucleolar state.

The existence of a certain degree of cross-immunity between N1 and the group N2 cell lines was established in confirmation of the data published by Sanford *et al.* (*2*). This can be interpreted as a result of an antigenic shift common to all these lines, at least with respect to the C3H/He mice utilized. However, the degree of this antigenic modification is very limited, as was demonstrated by the experiment in which N2 cells inoculated simultaneously with, but separately from, the cancer-producing cells did not influence the tumor growth. Their depressive effect, when admixed to the cancer cell inoculums, can be regarded as a result of complex local reaction phenomena.

Nevertheless, these results did not answer the essential question as to whether specific antigenic changes occur in direct connection with the increase of cancer-producing capacity of a cell population. Two possibilities are usually envisaged: additional antigens and/or loss of existing antigens.

No definite proof of the appearance of a new and specific antigen in the transformed high-cancer cell lines was produced, but the evidence of "enhanced" tumor growth after inoculation of the N2T′ cells in mice preimmunized with the same frozen cells was suggestive. Similarly, a certain degree of stimulation of these cells in mice by admixture of spleen-cell suspensions obtained from animals bearing old tumors was evidenced. However, further experiments are necessary to confirm these data.

The other possibility can be considered from the point of view of the delation theory (*11*). According to this theory, the N1 or the N2T lines would depend, for their tumor-producing capacity, on a *loss* of certain mechanisms or components through which their normal ancestors responded to "restraining and controlling influences" of the host. But it was proved by our experiments that the proliferative and invasive behavior of the tumor-producing N1, N2T′, and N2T^{bis} lines, but not of the low-cancer N2 line, could be demonstrated in such simplified tissue systems as the chorio-allantoic membrane and the chick-embryo mesonephros organ cultures. Mechanisms specifically controlling the growth of heterologous cells in these two rudimentary tissue systems are hardly imaginable, and immunological mechanisms can be excluded.

In this way, the malignant character of the derived cell lines, expressed by the proliferative infiltrating and invasive capacities of the cells, could be fully demonstrated outside the species proper to the cells. On the chorio-allantoic membrane, this character prevailed in the presence of a potent vascular reaction of a heterologous host as it did in the mesonephros organ cultures without the contribution of this reaction, and in both cases outside of any immunological interference.

In the light of these facts, antigenic simplification cannot be considered either as a sufficient or necessary explanation of the malignant growth. The capacity to produce tumors appears in the cell lines under study as a new characteristic *per se*, acquired and maintained by the cells through successive generations, and demonstrable outside the specific host-cell interaction.

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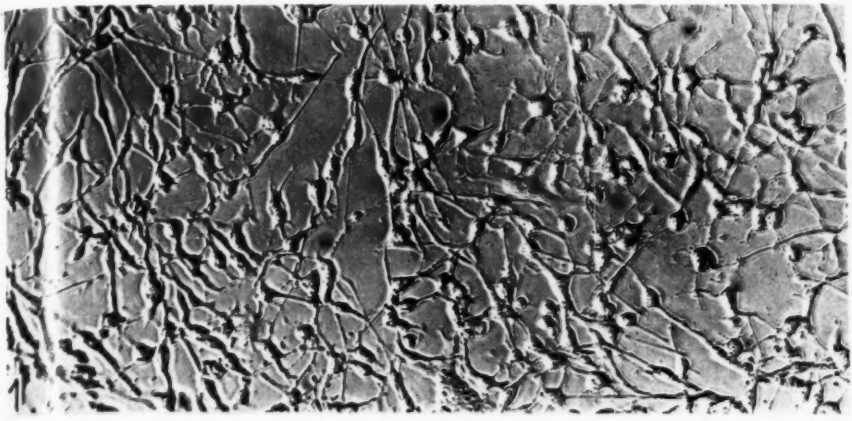
PLATES

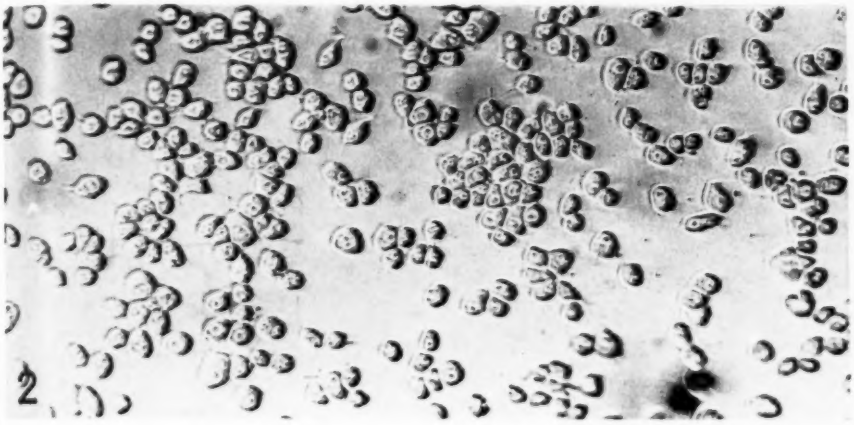
PLATE 107

FIGURE 1.—Cells of the high-cancer N1 line, NCTC clone 2472. Living culture. × 300

FIGURE 2.—Cells of the low-cancer N2 line, NCTC clone 2555. Living culture. × 300

FIGURE 3.—Cells of the derived N2T′ line. Living culture. × 300





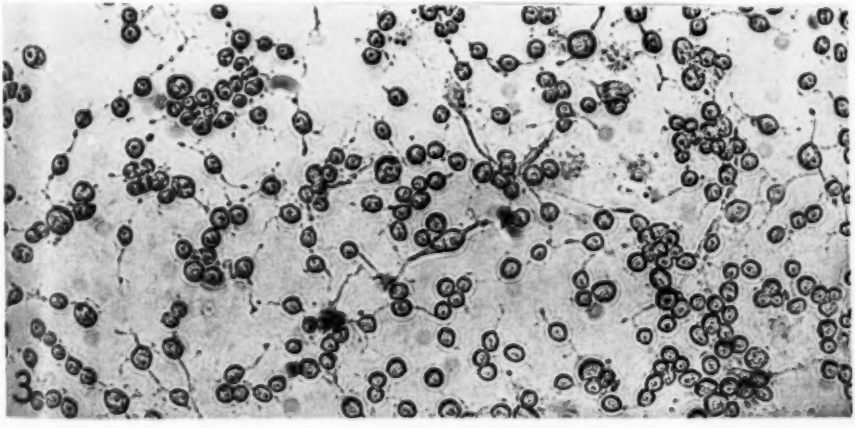


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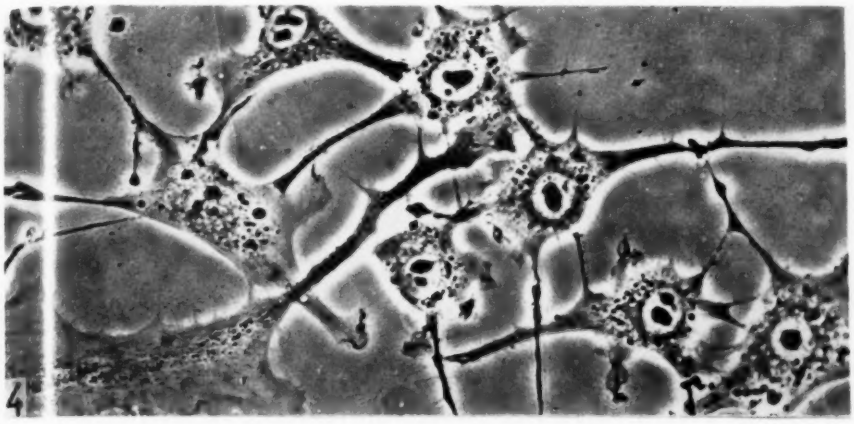
PLATE 108

FIGURE 4.—Cells of the high-cancer N1 line. Living culture, phase contrast. × 1,250

FIGURE 5.—Cells of the low-cancer N2 line. Living culture, phase contrast. × 1,250

FIGURE 6.—Cells of the derived N2T′ line. Living culture, phase contrast. × 1,250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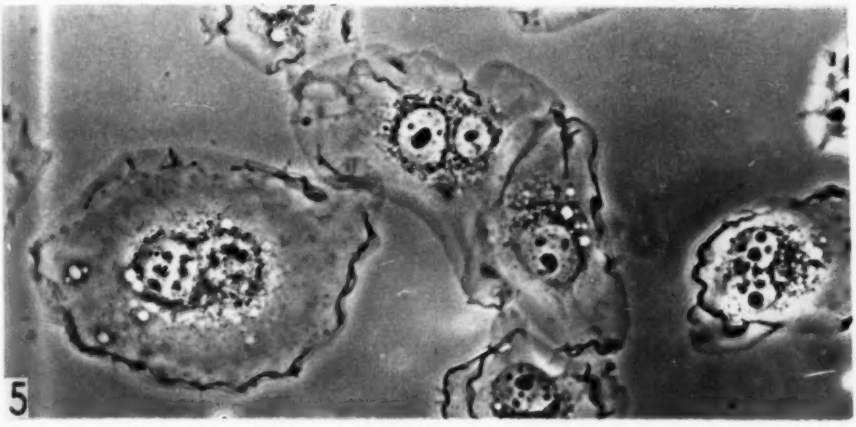
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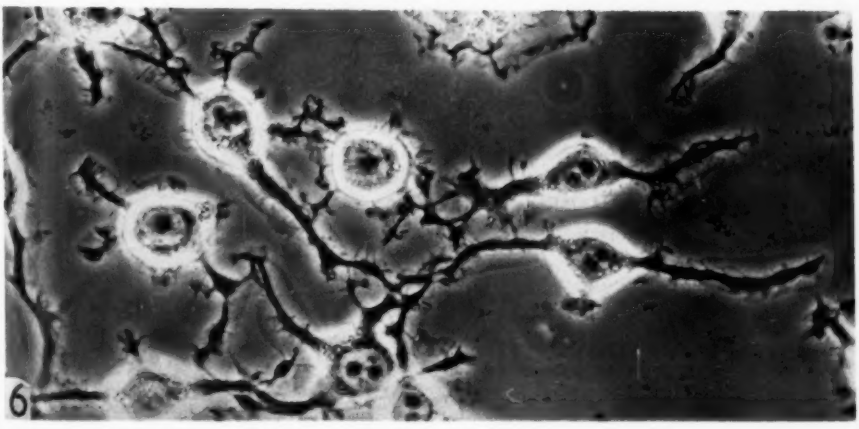
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PLATE 109

FIGURE 7.—High-cancer N1 line; 55 telocentric chromosomes, 1 strikingly lon Colchicine-treated metaphase, orcein-acetic acid staining. × 3,000 approx.

FIGURE 8.—N2 line; 57 chromosomes, 12 biarmed. Colchicine-treated metapha orcein-acetic acid staining. × 3,000 approx.

FIGURE 9.—Tumor-producing N2T′ line, derived from N2 line; 61 chromosome 11 biarmed. Colchicine-treated metaphase, orcein-acetic acid staining. × 3,000 approx.

FIGURE 10.—Tumor-producing N2T^{bis} line derived from N29; 63 chromosomes, 12 biarmed. Colchicine-treated metaphase, orcein-acetic acid staining. × 3,000 approx.

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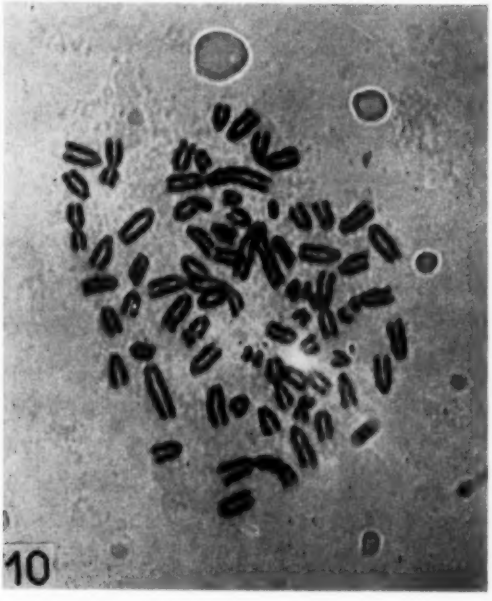
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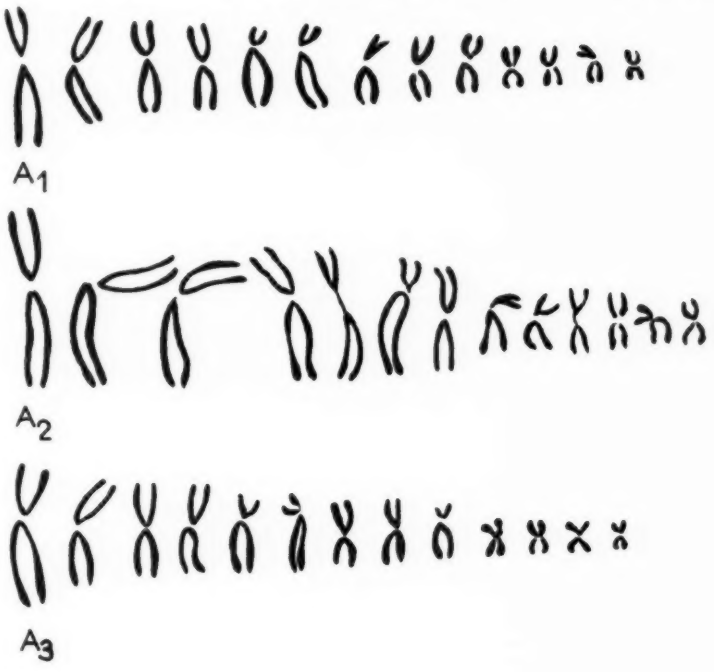
PLATE 110

FIGURE 11.—Idiograms of the biarmed chromosomes of the parental N2 and "derived" tumor-producing cell lines. Three different metaphases were analyzed for each line. A_1, A_2, A_3: N2 line. B_1, B_2: N2T′ line.



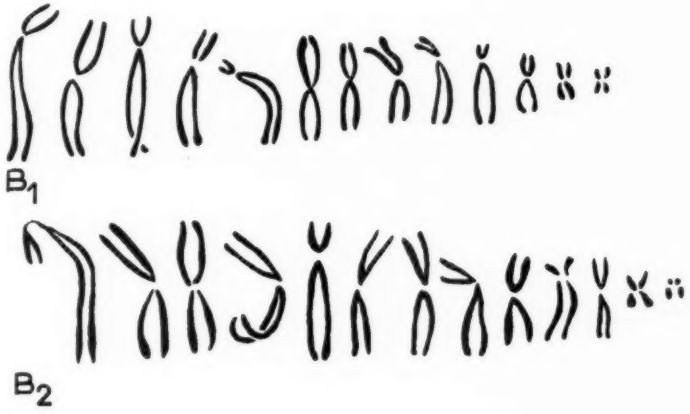


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PLATE 111

FIGURE 12.—Idiograms of the biarmed chromosomes of the parental N2 and "derived tumor-producing cell lines. Three different metaphases were analyzed for each line. B_3: N2T′ line. C_1, C_2, C_3: N2T^{bis} line.

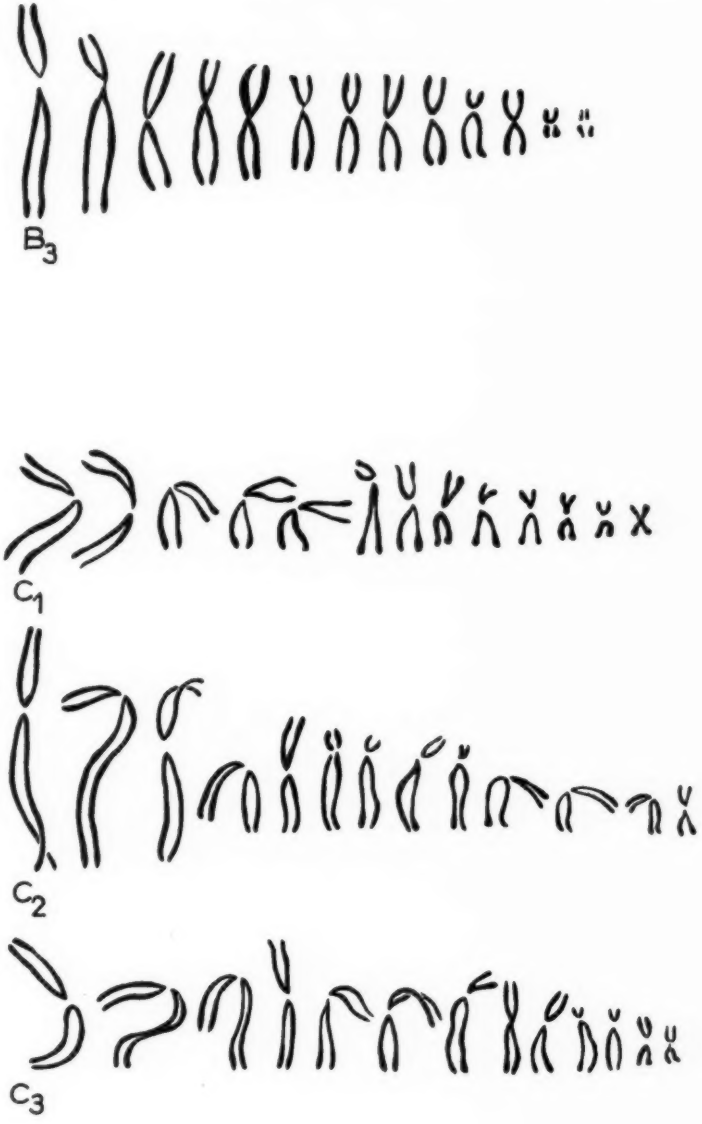

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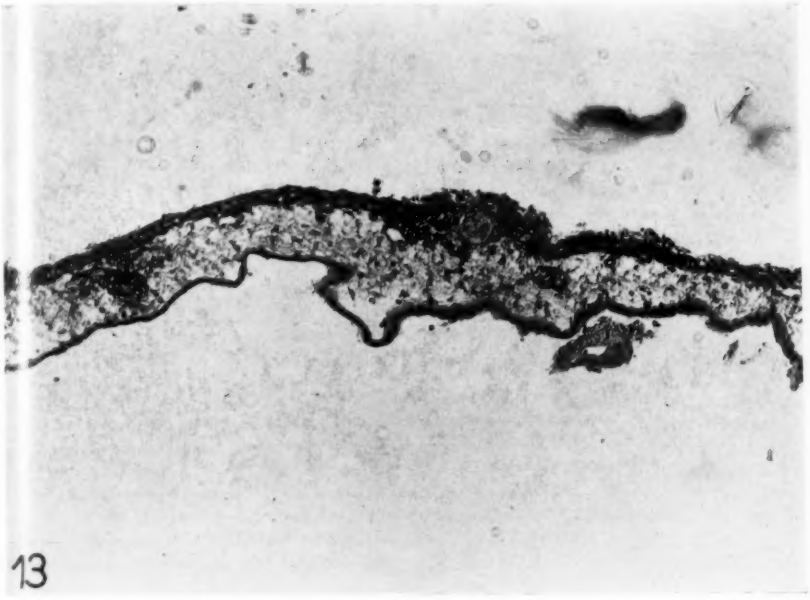
PLATE 112

Hematoxylin and eosin-stained sections of chorio-allantoic membranes, 8 days after inoculation with cell suspensions of N2, N1, N2T′ lines.

FIGURE 13.—Low-cancer N2 line. No growth, few degenerated cells on surface. × 130

FIGURE 14.—High-cancer N1 line. Extensive growth of well-vascularized, spindle-cell tumor nodule. × 130

FIGURE 15.—"Derived" N2T′ line. Well-vascularized, round-cell tumor nodule. × 130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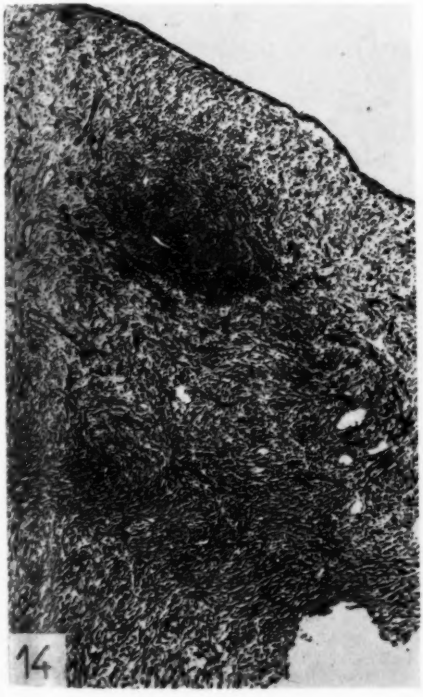
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A Transplantable Bile-Secreting Hepatocellular Carcinoma in the Rat [1, 2, 3]

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SUMMARY

A bile-secreting, transplantable hepatocellular carcinoma, with bile plugs in the canaliculi, is described in the rat. All transplants of this tumor, which has been carried for 6 generations, have shown bile pigment. This finding has not been previously reported. The tumor also contained iron and ceroid pigments. Bile pigment and iron were also present in the kidneys, in addition to a third unidentified pigment, which was probably lipofuscin.—J. Nat. Cancer Inst. 26: 891–897, 1961.

BILE SECRETION into the canaliculi between adjacent liver cells has frequently been noted in primary human hepatocellular carcinoma, but curiously, it is more often found in the metastases. It has also been noted in well-differentiated liver cells in a metastatic teratocarcinoma of the testis in a supraclavicular lymph node (*1*). The morphology of induced hepatic tumors in rats has been described in detail by various workers (*1–10*); however, the finding of bile pigment in these tumors has not to our knowledge been reported. Although this finding has been attributed to Kinosita (*2*), bile pigment is not described in his paper which has been quoted by others (*1*, *4*). Edwards and White (*4*) observed material suggesting intercellular biliary casts which were not pigmented in unstained sections and were often intimately associated with collagen. They concluded that these were strands of connective tissue that for some reason did not take the specific stains.

In the present work on liver carcinogenesis with the use of *N*-2-fluorenyldiacetamide, a semisynthetic diet, and A × C strain rats, several hundred primary hepatocellular carcinomas have been studied. Approximately 50 of these were transplanted isologously, homologously, or heterologously, each for 3 or more generations (*11*).

Many bile casts within canaliculi were observed in 1 line and smaller amounts of bile pigment in 2 other lines of transplanted hepatocellular carcinoma. The former is described in detail here.

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[2] Aided by a grant from the American Cancer Society, Inc.

[3] This research was started at the University of Kansas and completed at the University of Maryland, the *present address* of the author.

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HISTORY OF TUMOR

This bile-secreting tumor arose in a male A × C rat, which received 0.025 percent *N*-2-fluorenyldiacetamide in the semisynthetic diet of Morris, No. 272 (*12*). The diet consisted of 300 g commercial casein, 2275 g skim-milk powder, 6152 g ground, hard spring wheat, 200 g Brewer's yeast, 200 g desiccated whole liver powder (Wilson), 140 g sodium chloride, 10 g ascorbic acid, 13 g ferric citrate, 100 g cod-liver oil, and 610 g corn oil. The carcinogenic diet was administered for 4 weekly periods, each separated by 1 week of the same diet without carcinogen. Thereafter the rat was continued on the basal diet until autopsy was performed 10 months later. At the time of autopsy the rat weighed 269 g and the liver weighed 32.4 g. There was a 3.4 × 2.6 × 3.1 cm gray, nodular, firm tumor in the left lobe of the liver. Microscopic study disclosed a well-differentiated hepatocellular carcinoma with invasion of the portal vein and many metastases to the lungs. There was minimal cirrhosis in the liver and a few small cysts were seen. Multiple sections failed to reveal bile pigment within the tumor or in the pulmonary metastases.

The hepatoma that was transplanted subcutaneously to 3 male A × C rats grew in 2 of them. During the second generation it was transplanted into 3 female A × C rats and again grew in 2 rats. Bile plugs were observed in both the first and second transplanted generations. The tumor from one of the second-generation animals was labeled H-35 and was used in this study.

METHODS AND PROCEDURES

The third generation of the tumor was transplanted subcutaneously into the right groin of 25 A × C female rats 3 to 4 months of age, weighing 150 to 175 g. The rats were fed laboratory pellets. The animals were weighed and the tumors measured at 2- to 3-week intervals throughout the experiment. When the rats were killed at 4 months the serum and urine appeared icteric. The Glenner stain for bilirubin (*13*), and the Oil Red O stain for fat were performed on unfixed tumor and kidney. Tumor tissue was fixed in cold acetone, and alkaline-phosphatase studies were carried out according to the sodium glycerophosphate-calcium chloride technique of Gomori (*14*). The heart, lungs, kidneys, spleen, thymus, salivary gland, ovaries, adrenals, urinary bladder, and axillary, retroperitoneal, and groin lymph nodes were fixed in 10 percent formalin and stained with hematoxylin and eosin. The following stains were carried out on the tumor and kidney: periodic acid-Schiff (PAS), Verhoeff acid fast, Oil Red O, Sudan Black-B, Gomori iron, Stein bilirubin, Hall bilirubin (*15*), Glenner bilirubin, Masson's trichrome, Gridley's reticulum, and Kutlick's ferric-iron method for bilirubin.

RESULTS

At 1 month the transplants measured 0.2 to 0.5 cm in greatest diameter; at 2 months, 1.3 to 3.0 cm; at 3 months, 4.5 to 5.0 cm; and at 4 months, 5.0 to 6.5 cm. In 3 rats the transplant did not grow. The average weight of the animals at time of death was 106 g. The tumors varied in weight between 20 and 80 g but averaged 38 g.

Gross Findings

The tumors were usually cystic and this was evident by external palpation. The cystic areas measured up to 2 and 3 cm and contained reddish-brown or dark-purple fluid which was bile-stained. The tumor tissue resembled liver tissue partitioned by wide bands of darkly bile-stained connective tissue. Areas of the tumor 3 to 4 mm in diameter were also bile-stained, usually adjacent to the fibrous tissue (fig. 1). The corticomedullary junction of the kidney was brownish-green and the cortex was brown. After formalin fixation, the bile-stained areas of tumor and the corticomedullary junction of the kidney were bright green; the remainder of the cortex of the kidney was light green.

Microscopic Findings

The tumor had a trabecular pattern with double cords of cells separated by prominent vascular spaces which were often lined by Kuppfer cells. In areas it grew in solid sheets. There were many well-developed canalicular structures between adjacent tumor cells. Often these contained olive-green casts which in frozen sections were dark green (fig. 4). The cells of the tumor resembled hepatic parenchymal cells with abundant eosinophilic cytoplasm, central vesicular nuclei, prominent single nucleoli, and marginal chromatin lining the nuclear membrane (fig. 5). Intracytoplasmic inclusions were not observed. Reticulum was confined to perivascular areas and was negligible among tumor cells. The cystic areas were lined by compressed and atrophic tumor cells. Islands of tumor were separated by broad bands of collagen containing reticulum and scattered round macrophages with small nuclei. Tumor cells adjacent to these areas sometimes contained granules or small globules which were colored by Oil Red O in frozen sections but not in paraffin sections. PAS reaction revealed positive granules in the cytoplasm which were removed by previous diastase digestion.

Alkaline-phosphatase reaction was present in the sinusoidal linings and nuclei of Kuppfer cells by the Gomori technique. There was also very finely granular reactive material in the pericanalicular areas which served to identify those structures in many areas throughout the tumor. An incidental finding consisted of somewhat coarser granules in the cytoplasm adjacent to the sinusoids in occasional cells. They were not further identified.

Tumor cells appeared to be secreting small granules of olive-green pigment into the canaliculi (fig. 3). The olive-green casts in the canaliculi were always adjacent to the bands of connective tissue in the tumor. Nearby macrophages in the connective tissue also contained similar pigment. The pigment, which was light green or greenish-yellow on unfixed tissue, stained bright emerald green in unfixed tissue with the Glenner stain and in fixed tissue with the Hall stain. In unstained paraffin sections it was olive green. In paraffin sections with Kutlick's and Glenner's stains it deepened but remained olive green. It was negative for iron and isotropic between crossed polaroids.

Many macrophages contained coarse clumps of brown pigment which gave a positive reaction for iron. In others there was canary-yellow pigment, which was strongly acid-fast, PAS-positive, and fluorescent in ultraviolet light, and stained with Oil Red O both in frozen and paraffin sections.

Within the fibrous stroma large venules were surrounded irregularly by several layers of large tumor cells containing iron. Bile pigment was present within small canaliculi. Often these cells were separated from the lumen of the vessel by a single layer of endothelial cells. A few recent thrombi were noted within the vessels.

In the outer zone of the cortex of the kidney there were homogeneous spherical bodies in the cytoplasm and lumens of proximal tubules. These bodies varied from eosinophilic to olive green and measured 7 to 15 μ in diameter (fig. 2). They were bright yellow in unfixed frozen sections and light brown to olive in formalin-fixed frozen sections stained with Oil Red O. They appeared greenish yellow in unstained paraffin sections and pale green to olive with bilirubin stains.

There was also in the kidney brown pigment which often appeared clumped within the cytoplasm of many proximal tubular cells. In the inner zone of the cortex multiple brown droplets were observed in almost every cell lining the proximal and distal tubules. They remained brown with all stains. They occasionally stained faintly in fat stains of paraffin sections. Iron stains were negative.

In the cytoplasm of the proximal tubules in the outer zone of the cortex there were scattered, fine, iron-containing particles, which stained very light brown on hematoxylin and eosin. Brown clumps within stromal macrophages were also positive for iron.

There was extramedullary hematopoiesis and small amounts of hemosiderin in the pulp of the spleen. The regional lymph nodes contained hemosiderin and ceroid pigment within macrophages. In the lung of one animal there was a tiny metastasis. The remaining organs were not unusual.

DISCUSSION

Pigment studies have been carried out on the third-generation transplants of a well-differentiated hepatocellular carcinoma which acquired

the ability to secrete bile pigment in the first-generation transplant to an isologous host. In addition to olive-green bile casts, there were two additional pigments in the tumor. Both were predominantly within macrophages in the wide bands of connective tissue. One was in the form of granular, brown clumps which contained iron, apparently hemosiderin. The other pigment was in the form of somewhat clumped, canary-yellow, amorphous globules, and represented ceroid, an unsaturated lipide which had undergone auto-oxidation and polymerization. Often hemosiderin and ceroid were present within the same macrophages. There were also lipides within macrophages which did not remain after paraffin embedding.

The kidney contained three types of pigment. There were globules within the lining cells and lumens of the proximal convoluted tubules, particularly in the outer cortex. They were proportional to the number of bile plugs present within the tumor and are interpreted as bile pigment or bile-stained hyaline droplets. In the lining cells of the proximal convoluted tubules there were small scattered particles which gave a positive reaction for iron. There was also iron-containing material within the stromal macrophages. The third pigment, golden brown, granular material, was unidentified. It was observed within proximal convoluted tubular cells and stromal macrophages of the cortex. It seems likely that it was lipofuscin pigment.

Bile pigment has not been observed in primary or transplanted liver tumors in animals. Edwards and White (*4*) described both iron and ceroid pigment, but did not note bilirubin in their experiments with tumors in rats. The iron within macrophages in the tumor was explained on the basis of hemorrhage or hemolysis within the tumor. The ceroid that was at the periphery of the primary tumors was believed to be a remnant of the cirrhotic liver. Ceroid is present in experimental dietary cirrhosis in rats (*16–18*), in mice treated with carbon tetrachloride (*19*), in vitamin-E-deficient animals (*20*), and in fish affected with a wide variety of pathologic diseases (*21*); it can be produced *in vitro* by the mixture of unsaturated fats and red blood cells (*22*) or proteins (*23*).

Kinosita (*2*) observed iron within kidney tubular cells in rats with liver tumors. Fairhall and Miller (*24*) described pigment in the kidneys of rats receiving compounds of lead and concluded that it was de-ironized blood pigment. Similar pigment was reported by Edwards and White (*4*) in animals with hepatic tumors. It is also seen in kidneys of animals with other transplantable tumors.

Attempted correlation of the findings suggests that the bile pigment secreted by the tumor was carried in the blood stream and then excreted by either the liver or the kidneys, or both. It was present as globules within the proximal convoluted tubules of the kidney. Iron pigment from hemorrhage or hemolysis entered the circulation and, when the level was great enough in the blood, it could be identified within the kidney as such. Ceroid pigment probably did not enter the circulation but was carried in macrophages to regional lymph nodes.

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PLATE

Plate 113

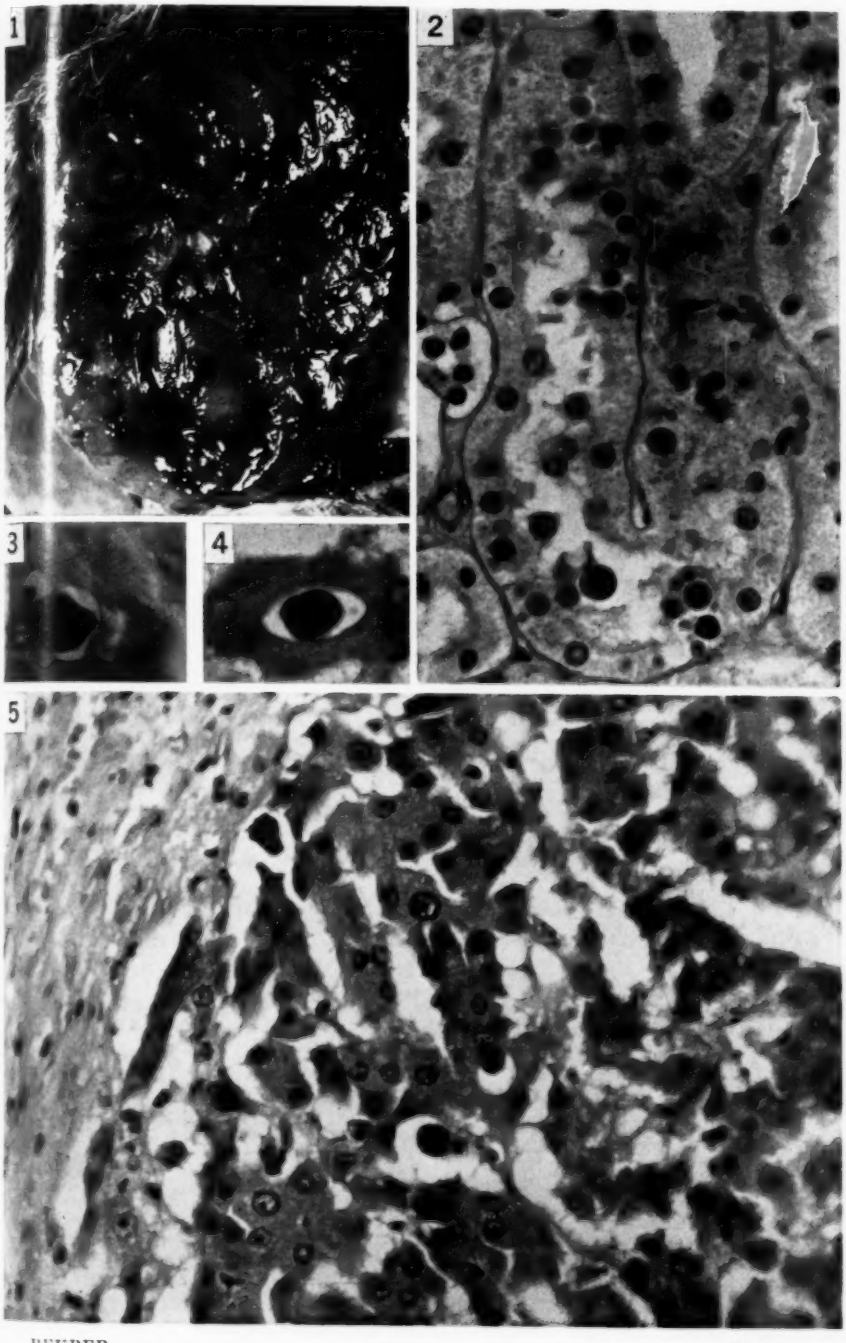
Figure 1.—Tumor transplant resembling liver tissue partitioned by wide bands of darkly green-stained connective tissue. Bile-stained areas of tumor tissue adjacent to connective tissue and cysts containing bile-stained fluid.

Figure 2.—Globules of bile pigment or bile-stained hyaline droplets within cell and lumen of proximal convoluted tubule of cortex of kidney with periodic acid-Schiff. × 700. These vary from brown to olive green and sometimes orange, with hematoxylin and eosin stains.

Figure 3.—Tumor cells with bile pigment within the cytoplasm, often surrounded by a clear halo, and a bile cast in the lumen. Hematoxylin and eosin. × 810

Figure 4.—Canalicular structure between adjacent tumor cells containing a bile cast. Hematoxylin and eosin. × 700

Figure 5.—Tumor cells resembling hepatic parenchymal cells; adjacent band of connective tissue containing many macrophages (not shown) with ceroid and iron pigment and few with bile pigment; bile casts within canaliculi. Hematoxylin and eosin. × 415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Effect of Foster Nursing on Morphology of Lymphatic Tissue of Mice [1, 2]

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SUMMARY

A quantitative morphological examination of the thymus, spleen, and left axillary lymph node of 6½- to 7½-week-old female mice of the high-leukemic PL/Sp and AKR/Sp and the low-tumor C57BL/Sp strains was carried out. Strain differences were noted in all the lymphatic tissues studied. Morphologically the spleens, but not the thymuses or lymph nodes, of PL/Sp and AKR/Sp mice were quite similar to one another and differed markedly from those of C57BL/Sp mice. The differences were interpreted to indicate that lymphopoiesis directly from reticulum cells occurred more frequently in spleens of PL/Sp and AKR/Sp mice than in spleens from C57BL/Sp mice. Lymphopoiesis from lymphatic elements, as judged by mitotic activity, was most pronounced in the thymuses of all mice studied. Foster nursing affected the morphology and the mitotic activity of all lymphatic tissues. Although a maternal influence exerted during the nursing period may have accounted for some of the strain differences in the lymph nodes, it could not be demonstrated as a factor causing differences in the spleen.—J. Nat. Cancer Inst. 26: 901–917, 1961.

STRAIN DIFFERENCES in murine lymphatic tissue have been recognized for some time (*1*). On comparison of mice from high- and low-leukemic strains, differences have been observed in the peptidase activity (*2*), the weight (*3*), and the lymphocytosis-stimulating-factor content of the thymus, the lymphocytic response to this factor, the resting lymphocyte levels (*4*), and the glycolytic activity of lymph nodes (*5*). Our interest in the lymphatic tissue of mice, prior to the development of neoplasia, began when it was found that the peripheral lymph nodes of virgin mice of the high-mammary cancer C3H strain weighed more and were morphologically more reactive than those from mice that were also susceptible to mammary-cancer development but had much lower incidences (*6*). A parallel study (*7*), in which only lymph-node weight was investigated, indicated that the nodes from 6½- to 7½-week-old

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female mice of the high-leukemic PL/Sp and AKR/Sp strains weighed more than the corresponding nodes from mice of the low-tumor C57BL/Sp strain. To extend these studies, a quantitative morphological investigation of the thymus, spleen, and lymph nodes from young virgin female mice of high-leukemic and low-tumor strains was undertaken.

The reasons for the previously described differences in lymphatic tissues of mice from high- and low-tumor strains could have been due to genetic factors and/or prenatal or postnatal environmental factors. Since previous investigations (7) had indicated that a maternal influence operative during the nursing period influenced lymph-node size, the effect of foster nursing on the morphology of the lymphatic system was also investigated.

MATERIALS AND METHODS

Virgin female mice of the high-leukemic AKR/Sp and PL/Sp and the low-tumor C57BL/Sp strains,[3] hereinafter referred to as AKR, PL, and C57, were kept in a metal or wooden cage on racks, 5 to 10 animals per cage, in an air-conditioned room and fed Purina laboratory chow and water *ad libitum*. The mice were inspected daily and animals in poor health were discarded. Cages were changed weekly. Mice to be foster nursed were females obtained by first-generation pen-breeding. PL and C57 sucklings, as representatives of a high-leukemic and of the low-tumor strain, respectively, were used in the foster-nursing experiments. The newborn mice remained with their mother up to 18 hours before being transferred to foster mothers. All animals were weaned at 5 weeks and killed at 6½ to 7½ weeks of age by cervical dislocation. All animals were weighed prior to being killed. A thoracic and abdominal autopsy was performed on each animal. The 2 elbow (brachial) and 2 inguinal lymph nodes (6 and 1, fig. 7) and the spleen were removed, blotted, and weighed on a Roller-Smith torsion balance. A block of tissue from the midportion of the spleen, the entire thymus, and the left axillary node were taken for microscopic examination. The tissues were fixed in Bouin's solution, sectioned at 6 μ, and stained with hematoxylin and eosin. The left axillary lymph nodes were cut parallel to their flat surfaces and sections for study were taken from the largest diameter of each node. Splenic and thymic tissue blocks were cut at right angles to their long axes and the largest sections were taken for study. With a microscope graticule,[4] the total area of the section and the regions that were principally occupied by sinus reticulum cells, connective tissue, medullary cords, intermediate zone, and lymphatic nodules in the lymph nodes were determined in the following manner: At a magnification of 32× each section was divided

[3] These mice were obtained from the inbred stocks maintained in the Detroit Institute of Cancer Research. In this colony the incidence of leukemia in virgin female mice of the AKR/Sp and PL/Sp strains is about 80 and 60 percent, respectively. The total tumor incidence in female mice of the C57BL/Sp strain is less than 10 percent.

[4] The microscope graticule was purchased from James Swift and Son, Ltd., 113-115a Camberwell Rd., London, England, and bore the catalog number ME17.

vertically into segments of 0.125 mm, and each vertical segment was then measured horizontally in mm. The segments thus measured for the entire section and for each structure of the section were totaled individually and the total was multiplied by 0.125 mm to derive the area. The area principally occupied by a particular type of tissue was calculated as a percent of the whole section. The area occupied by the white pulp, red pulp, and the trabeculae of the spleen and by the lymphatic and reticular tissue of the thymus was determined in a similar manner; due to the size of the sections from the latter two organs, each section was divided into segments of 0.313 mm. These measurements are presented in tables 1 to 4 as percentages of the total area of the sections.

To estimate mitotic activity, the number of mitoses that occurred in each structure in a given section was counted at a magnification of 450× and then divided by the area occupied by that component to give the number of mitoses per mm^2 of a given structure that is shown in the tables. A nucleus was considered in mitoses from the time definite chromatin threads were observed until membranes had formed in the daughter nuclei.

RESULTS

Since the body weights and lymph-node weights have been reported previously (7) and in no way bear on the results presented herein, they were not included. The spleen weights have not been reported previously and are included in this presentation.

Thymus

The area occupied by the lymphatic tissue in thymuses of AKR mice was larger and that occupied by the reticular tissue was smaller than the corresponding areas in thymuses of either PL or C57 mice (table 1). Fewer mitoses were found among the lymphocytic elements of PL mouse thymuses than among the lymphocytic elements of thymuses of AKR and

TABLE 1.—Structure and zonal distribution of mitosis in thymuses of mice 6½ to 7½ weeks of age*

Strain	Foster mother	Number of mice	Lymphatic tissue		Reticular tissue	
			Percent	Mitosis	Percent	Mitosis
PL	—	18	72. 0(1. 2)†	284(16)‡	28. 0(1. 2)	4. 6(0. 5)
AKR	—	18	85. 8(1. 3)	411(16)	14. 2(1. 3)	8. 4(1. 0)
C57	—	18	71. 6(1. 7)	392(27)	28. 4(1. 7)	14. 5(2. 7)
PL	PL	18	72. 0(1. 9)	307(15)	28. 0(1. 9)	10. 6(0. 6)
PL	C57	13	69. 5(1. 8)	302(13)	30. 5(1. 8)	8. 7(0. 5)
C57	C57	18	80. 2(1. 8)	328(26)	19. 8(1. 8)	22. 9(2. 5)
C57	PL	14	81. 6(1. 4)	311(21)	18. 4(1. 4)	19. 2(4. 2)

*Figures represent means and their standard errors.
†Figures for organ structures are percentages of the area studied.
‡Figures for mitoses represent the number of mitoses per mm^3 of structure.

C57 mice. Fewer mitoses were observed in the reticular tissue of thymuses of mice from both PL and AKR strains than in this component of C57 mouse thymuses.

While some change in the thymus resulted from foster nursing, this change was not always the same (table 1). When PL suckling mice were reared by either PL or C57 foster mothers, there was an increase in the number of mitoses found in the reticular tissue. When C57 mice were reared by either C57 or PL nurses, the area occupied by the lymphatic tissue was increased, while that occupied by the reticular tissue was correspondingly decreased. Mitotic activity in the various areas of the thymus was also altered in foster-nursed C57 mice. However, the area affected depended on the foster mother. When C57 nurses were used, there was a significant increase in the number of mitoses found in the reticular tissue. On the other hand, when PL foster mothers were used, the number of mitoses in the lymphatic tissue was significantly diminished.

Spleen

The spleens from nonfoster-nursed mice of the PL and AKR strains weighed significantly less than those from mice of the C57 strain (table 2). This was not due to differences in body weight since the spleen to body-weight ratios were also significantly different.

TABLE 2.—Composition of spleens of mice 6½ to 7½ weeks of age*

Strain	Foster mother	Number of mice	Spleen weight (mg)	Spleen ratio†	Red pulp	White pulp	Trabeculae
PL	—	17	43.1(1.6)	2.8(0.1)	43.1(1.2)‡	55.8(1.2)	1.2(0.2)
AKR	—	17	53.3(8.1)	3.6(0.6)	50.5(1.4)	48.0(1.3)	1.5(0.2)
C57	—	18	78.1(3.9)	5.3(0.3)	65.7(0.9)	32.6(0.9)	1.7(0.2)
PL	PL	17	51.1(4.5)	3.6(0.4)	44.9(1.9)	54.2(1.9)	0.9(0.2)
PL	C57	15	46.8(2.4)	2.8(0.1)	45.6(2.0)	53.4(2.1)	1.0(0.2)
C57	C57	18	57.4(5.0)	4.5(0.3)	48.9(1.8)	49.4(1.6)	1.7(0.3)
C57	PL	16	73.4(6.8)	5.1(0.4)	50.8(2.1)	47.6(2.2)	1.7(0.2)

*Figures represent means and their standard errors.
†The figures in this column are ratios of organ weights (mg) to body weights (g).
‡Figures for organ structures are percentages of the area studied.

Structurally, spleens from mice of the PL and AKR strains differed markedly from those of C57 mice (figs. 1, 2, and 5). Lymphatic nodules were more abundant and their centers were more distinct in the spleens of mice from the latter strain. On the other hand, spleens from mice of the PL and AKR strains contained many prominent subcapsular, dark-staining areas. These areas were composed of aggregates of small cells, with dark-staining nuclei and scant cytoplasm, that appeared to be lymphocytes and were considered as such. Along with these were small groups of cells with denser nuclei, occasionally possessing a small rim of eosinophilic cytoplasm, that were considered to be nucleated red blood cells. Cellular

forms that appeared to be intermediate between the small, dark cells and reticulum cells were also found (figs. 3 and 4). Insofar as these cellular aggregates appeared, by morphological criteria,[5] to consist principally of lymphocytes, they were included along with the white pulp. Such cells were almost entirely lacking in the subcapsular area of spleens from C57 mice (fig. 7).

That larger spleens were associated with one type of pattern and smaller spleens with another suggests that splenic structure and size are related. To test this possibility, spleens from C57 mice, large spleens with one type of pattern, were divided into two groups. One contained spleens that weighed 45.5 to 67.0 mg (5 animals) and the other, spleens that weighed 74.3 to 117.7 mg (12 animals), and the patterns were compared. The smaller spleens contained 31.2 percent white pulp, 66.6 percent red pulp, and 2.2 percent trabeculae. The larger spleens were made up of 33.8 percent white pulp, 64.8 percent red pulp, and 1.4 percent trabeculae. Spleens from mice of the AKR strain, small spleens with another type of pattern, were also divided into two groups according to their weights. Those that weighed 23.4 to 42.0 mg (8 animals) consisted of 45.5 percent white pulp, 53.0 percent red pulp, and 1.5 percent trabeculae. Those that weighed 47.4 to 176.0 mg (10 animals) contained 52.0 percent white pulp, 46.6 red pulp, and 1.4 percent trabeculae. It would seem, therefore, from these data, that splenic pattern was independent of those factors concerned with size.

The red pulp of spleens from PL and AKR mice contained more mitoses than did that of spleens from C57 mice. On the other hand, mitotic activity was more pronounced in the white pulp of the latter (table 4). Mitotic activity in the white pulp of PL and AKR mouse spleens occurred predominantly in the lymphatic nodules and was infrequently observed in the subcapsular lymphocytic aggregates.

When PL sucklings were reared either by PL or C57 nurses, no alteration in splenic size or pattern was observed. Mitotic activity in both white and red pulp was increased with PL nurses but not with C57 foster mothers (table 4). This indicated that the milk of PL mothers contained materials that were more stimulating than were the components of C57 milk.

When C57 nurslings were reared by either C57 or PL nurses, the splenic pattern was significantly altered in two ways: 1) The area occupied by the red pulp was decreased while that occupied by the white pulp was increased (table 2). 2) The qualitative appearance of the two types of tissue was changed. There was a decrease in the number of lymphatic nodules in the white pulp (fig. 6). In the red pulp many prominent subcapsular, dark-staining areas were observed (fig. 6). These areas were composed of aggregates of small cells with dark-staining nuclei and scant cytoplasm (fig. 8). These cells were similar in appearance to those found in the spleens from nonfoster-nursed PL and AKR mice. Cellular forms

[5] We wish to thank Dr. Bjarne Pearson, pathologist at the Detroit Institute of Cancer Research, and Dr. Thelma B. Dunn, Laboratory of Pathology, National Cancer Institute, for help in distinguishing these cells.

intermediate between the small, dark cells and reticulum cells were also seen (fig. 8). There occurred concomitantly a significant increase in the mitotic activity of the red pulp (table 4).

Lymph Nodes

Although strain differences were found in the composition (table 3) and the microscopic appearance (figs. 9, 10, and 11) of the lymph nodes of nonfoster-nursed mice, there was no particular pattern that was common to mice of the high-leukemic PL and AKR strains. Lymph nodes from mice of the PL strain were characterized by a large lymphatic nodule area, a moderate medullary-cord area, and a small intermediate zone (fig. 9). Those from mice of the AKR strain had a small lymphatic nodule area and a large medullary-cord area and intermediate zone (fig. 10). Nodes from C57 females were characterized by moderate lymphatic nodules and medullary cord areas and a large intermediate zone (fig. 11).

TABLE 3.—Composition of lymph nodes of mice 6½ to 7½ weeks of age*

Strain	Foster mother	Number of mice	Sinus reticulum cells	Fibrosis	Medullary cords	Intermediate zone	Lymphatic nodules
PL	—	17	9. 6(1. 0)†	0. 9(0. 5)	24. 9(2. 4)	10. 7(3. 2)	53. 8(3. 9)
AKR	—	14	5. 6(0. 7)	0. 9(0. 5)	46. 4(2. 9)	38. 3(3. 1)	8. 7(1. 1)
C57	—	14	4. 9(1. 0)	5. 4(2. 1)	20. 6(2. 0)	50. 6(3. 0)	18. 5(2. 5)
PL	PL	14	7. 0(0. 7)	0. 1(0. 1)	30. 2(2. 3)	4. 1(1. 0)	58. 6(2. 4)
PL	C57	14	6. 9(0. 7)	3. 2(1. 5)	27. 1(2. 3)	6. 9(1. 4)	55. 9(2. 4)
C57	C57	8	7. 8(1. 6)	1. 1(0. 7)	27. 3(3. 1)	44. 9(3. 6)	19. 0(2. 5)
C57	PL	12	6. 4(0. 9)	2. 2(1. 1)	32. 2(3. 1)	33. 7(4. 8)	25. 5(3. 4)

*See footnote, table 1.
†See footnote, table 1.

Mitotic activity was most pronounced in the medullary cords of lymph nodes from nonfoster-nursed mice of all three strains (table 4). It was reduced when PL mice were reared by C57 nurses, but was not affected when C57 mice were brought up by foster mothers. Mitoses were more frequent in the lymphatic nodules of mice of the PL strain than in the corresponding areas of nodes from mice of the other strains. The increased mitosis was apparently due, primarily, to some factor in the maternal milk, since foster nursing to C57 mothers reduced it and the reverse combination increased it (table 4). Although mitotic activity in the lymphatic nodules and medullary cords could be altered by foster nursing, the distribution of the various structures within the nodes remained unchanged (table 3). This indicated that inherited factors may be responsible for determining the pattern of lymph-node structure.

In our experiments, the production of lymphocytes from lymphatic elements, homoplastic lymphopoiesis, as indicated by mitotic activity, was most pronounced in the thymuses of mice of all strains studied. This is in agreement with the findings of Kindred (*8*) that in rats, at an early age, mitosis was more frequent in the thymic cortex than in either the lymph-node cortex or in the lymphatic cords of the spleen.

TABLE 4.—Zonal distribution of mitosis in spleens and lymph nodes of mice 6½ to 7½ weeks of age*

Strain	Foster mother	Spleen		Lymph nodes		
		White pulp	Red pulp	Lymphatic nodules	Medullary cords	Intermediate zone
PL	—	7. 5(1. 3)†	17. 6(1. 8)	18. 7(2. 2)	20. 6(4. 1)	4. 3(1. 1)
AKR	—	11. 2(2. 0)	14. 7(1. 7)	6. 4(1. 9)	14. 1(5. 6)	0. 6(0. 3)
C57	—	30. 2(2. 1)	11. 0(1. 3)	7. 3(2. 5)	20. 7(4. 5)	2. 1(0. 7)
PL	PL	17. 1(1. 9)	24. 7(2. 7)	14. 1(2. 1)	16. 6(2. 3)	0. 7(0. 5)
PL	C57	11. 5(1. 9)	18. 4(3. 7)	2. 5(0. 7)	9. 0(1. 9)	4. 6(3. 6)
C57	C57	27. 2(2. 7)	18. 1(1. 4)	5. 8(2. 3)	18. 0(3. 8)	7. 9(2. 1)
C57	PL	32. 1(5. 4)	21. 7(3. 3)	27. 1(5. 4)	22. 1(3. 1)	5. 5(1. 1)

*Figures represent means and their standard errors.
†Figures for mitoses represent the number of mitoses per mm.^{2} of structure.

DISCUSSION

The spleens from nonfoster-nursed PL and AKR and foster-nursed C57 mice contained less red pulp and more white pulp than did those from nonfoster-nursed C57 mice. The increase in the white pulp was due to the inclusion, as white pulp, of subcapsular aggregates of small cells with dark-staining nuclei and scant cytoplasm. Although these areas contained small groups of nucleated red blood cells, they consisted principally of lymphocytes. The presence of these lymphocytes could be attributed to deposition of lymphocytes manufactured elsewhere, *in situ* homoplastic lymphopoiesis or derivation directly from the reticulum cells of the red pulp, heteroplastic lymphopoiesis. Our findings of a scarcity of mitotic activity in the subcapsular lymphocytic aggregates, of cells intermediate between reticulum cells and lymphocytes within these aggregates, and of the association of their appearance in spleens of foster-nursed C57 males with an increased mitosis in the red pulp favored a direct derivation of lymphocytes from reticulum cells. Other workers (*9–11*) have also found instances in which lymphocytes seem to be derived directly from reticulum cells.

The reason for the presence of large numbers of subcapsular aggregates of lymphocytes in the spleens of mice of the high-leukemic PL and AKR strains was not immediately obvious. Our data indicated that they normally did not occur to any extent in the spleens of mice of the low-tumor C57 strain at the age studied. Since they could be induced by foster nursing, they may be a response to an increased requirement for lymphocytes in this organ. This suggestion is supported by the observation of Wissler (*11*) who found a similar process in the spleens of rats after antigenic stimulation. It would seem possible, therefore, that these aggregations in the spleens of mice of the PL and AKR strains might also be due to the presence of some, as yet unrecognized, type of stimulation. An alternative explanation is that the "normal" need for lymphocytes in the spleens of PL and AKR mice was met by a combina-

tion of homoplastic lymphopoiesis and subcapsular aggregates of lymphocytes due to some insufficiency in the former. This latter concept is supported by the low mitotic activity in the splenic white pulp of these animals.

It was quite clear from the data that the splenic differences observed in these experiments were not due to a maternal influence exerted during the nursing period, though it did account for a large portion of the mitotic activity in the lymphatic nodules and medullary cords of the lymph nodes from PL mice. It seems, therefore, that they were most likely due to inherited factors. This possibility is currently under investigation.

It was rather surprising to find that intrastrain foster nursing was accompanied by changes in the thymus and spleen. This suggested that the suckling must have become somewhat conditioned, during its *in utero* sojourn, to materials that were similar to ones that appeared later in the milk of its own mother.

The present findings contribute additional evidence that the development of leukemia in mice may be preceded by alterations in some of the lymphatic tissues that are recognizable early in life prior to the onset of the actual disease. It remains to determine how these changes are related to the development of leukemia and what is responsible for them.

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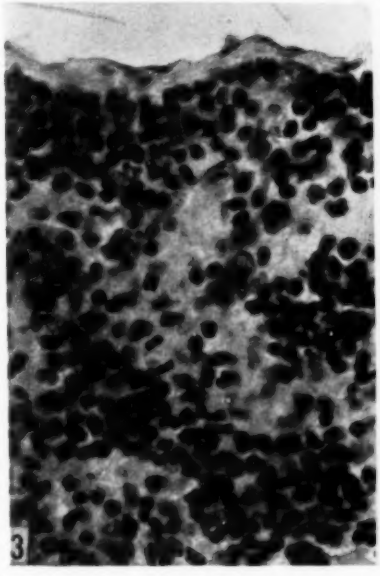
PLATES

583178—61——14

PLATE 114

FIGURES 1 AND 2.—Spleens from PL (fig. 1) and AKR (fig. 2) mouse. *Note* relatively few lymphatic nodules with indistinct secondary centers and the numerous prominent, subcapsular, dark-staining areas. × 34

FIGURES 3 AND 4.—Cellular pattern of the subcapsular region of spleens from PL (fig. 3) and AKR (fig. 4) mouse. *Note* numerous small cells, with dark-staining nuclei and sparse cytoplasm, most of which are lymphocytes (*see* text), cellular forms intermediate between these cells and reticulum cells, and relatively infrequent mitosis. × 340



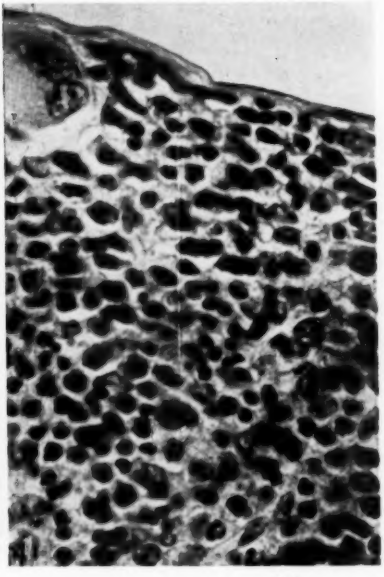


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PLATE 115

FIGURE 5.—Spleen from C57 mouse. *Note* numerous lymphatic nodules with distin[ct] secondary centers and relatively infrequent and not very prominent subcapsula[r] dark-staining areas. × 34

FIGURE 6.—Spleen from C57 mouse reared by C57 foster mother. This pattern [is] characteristic of spleens of all foster-nursed C57 mice studied. It shows a decrea[se] in lymphatic nodules and appearance of numerous prominent subcapsular, dark-staining areas. × 34

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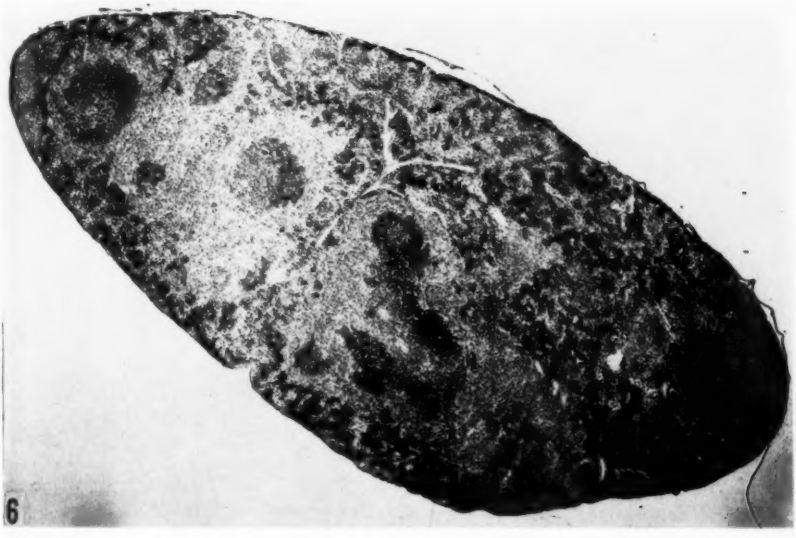
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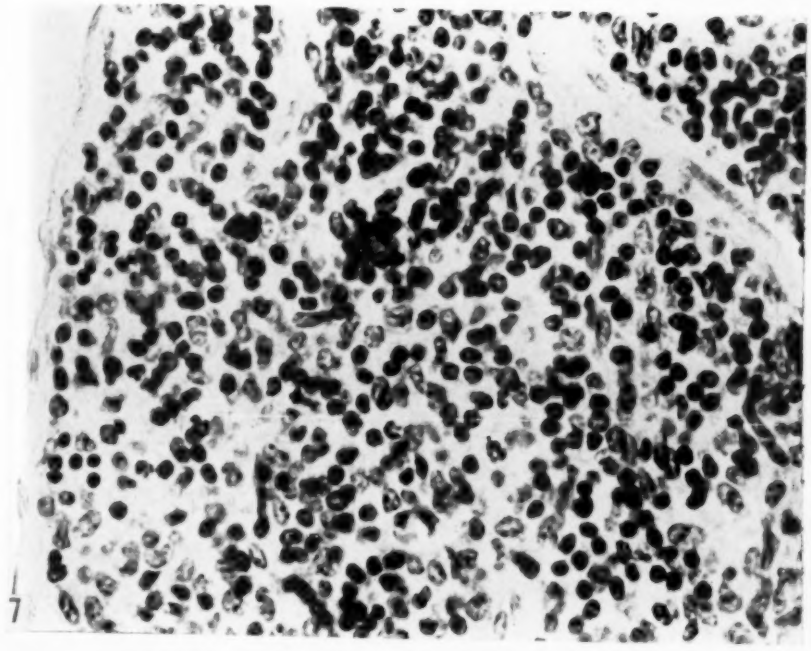
PLATE 116

FIGURE 7.—Cellular pattern of subcapsular region of spleen from C57 mouse. *Note* scarcity of subcapsular lymphocytes with dark nuclei and of cellular forms intermediate between them and reticulum cells. × 340

FIGURE 8.—Cellular pattern of subcapsular region of spleen from C57 mouse reared by C57 nurse. This cellular pattern is characteristic of spleens from all foster-nursed C57 mice that were studied. It shows the presence of numerous small cells, with dark-staining nuclei and scant cytoplasm, most of which are lymphocytes (*see* text), cellular forms intermediate between them and reticulum cells, and relatively infrequent mitosis. × 340



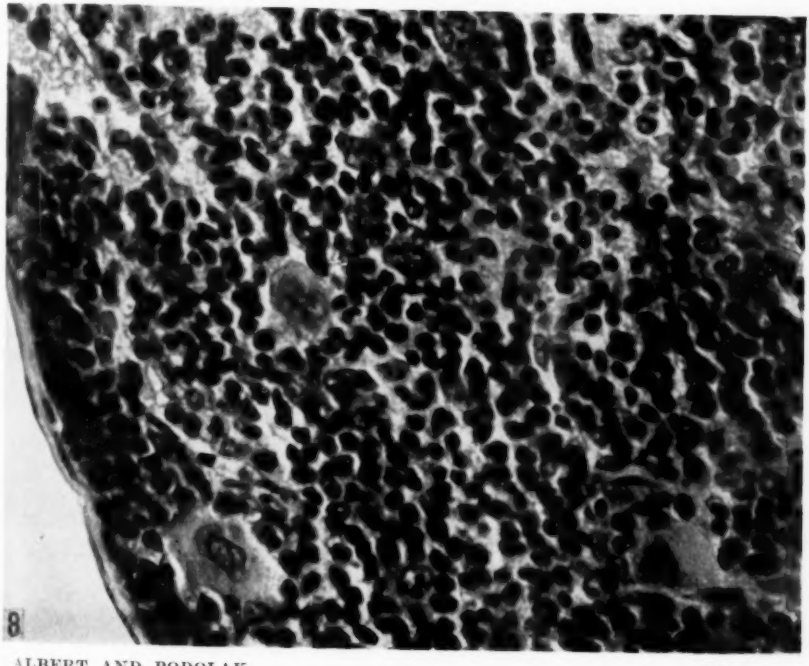
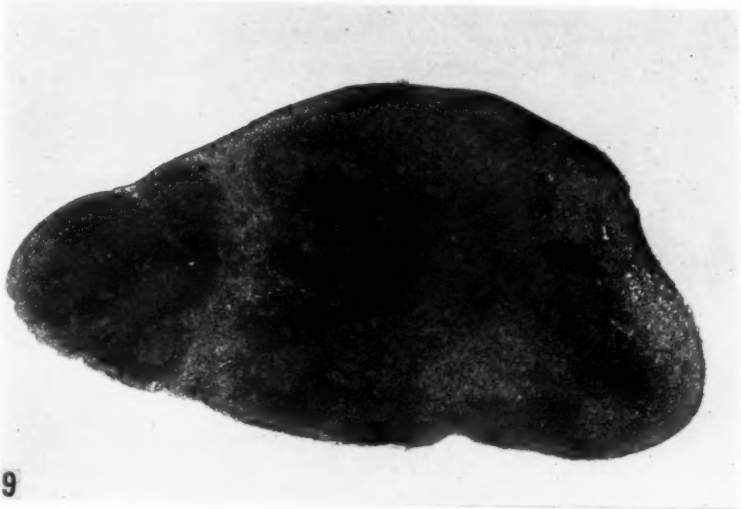


PLATE 117

FIGURE 9.—Lymph node from PL mouse. *Note* lymphatic nodules with their prominent secondary centers. × 40

FIGURE 10.—Lymph node from AKR mouse. *Note* prominent medullary cords and intermediate zone. × 40

FIGURE 11.—Lymph node from C57 mouse. *Note* prominent intermediate zone. × 65



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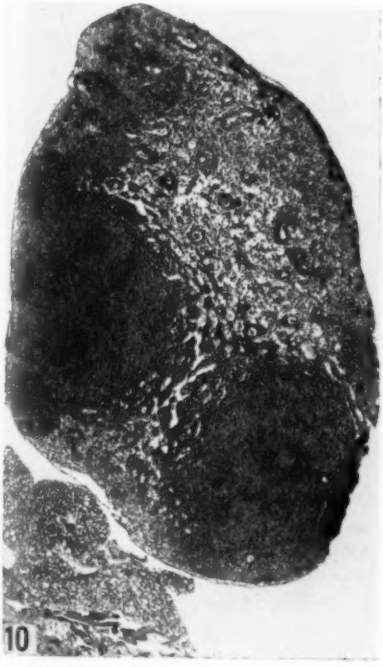

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Electron Microscopy of Tissue of the Mammary Glands and Tumers in Old Mice With Special Reference to Mitochondrial Size [1]

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SUMMARY

Mammary-tumor tissue as compared with hyperplastic mammary-gland tissue was studied with the electron microscope in old mice with the milk factor. Special attention was paid to differences in size and average diameter of mitochondria in tumor tissue and in cells of mammary tissue without signs of malignant transformation. In tumor cells the average mitochondrial diameter was consistently lower than in mammary-gland-tissue cells. A similar difference was found between mitochondria in a small tumor and those of the surrounding mammary-gland tissue in which the tumor had originated. Virus-like particles were present in tumor cells, but usually in smaller numbers than in cells of hyperplastic mammary tissue. In acini of hyperplastic mammary-gland tissue, in which a tumor had originated, the number of particles was extremely large. Iron-containing pigment probably consisting partly of ferritin was abundantly present in hyperplastic mammary tissue, whereas in tumor cells this pigment was practically nonexistent.—J. Nat. Cancer Inst. 26: 919–947, 1961.

IT IS generally assumed that, in the genesis of mammary carcinoma in high-tumor strains of mice, several factors are significant: 1) a genetic susceptibility; 2) hormonal influences (*1*, *2*); 3) environmental factors (*3*); and 4) a virus-like extrachromosomal agent which is transmitted during lactation (*4–6*). Hence the mammary-tumor agent is present during most of the animal's lifespan. Biological methods have shown the presence of the agent in mammary tumors, in mammary tissue without malignant transformation, and in several other organs. The electron microscope revealed characteristic round or oval bodies in thin sections of mouse mammary carcinoma (*7*, *8*). These particles possibly are viral in nature and probably represent the mammary-tumor agent. There seem to be two types of particles. One type occurs in the cytoplasm of the tumor cells and shows an electron-optically, relatively clear, homogeneous core surrounded by a zone consisting of two lamellae with a less electron-dispersing interspace, the average diameter of the particles being about 700 A (*8*). The second type occurs outside the cell and shows

[1] Received for publication October 18, 1960.
[2] We wish to thank Mr. W. Beens for his valuable technical assistance.

round or compressed particles which have a central, less electron-dispersing area with a more strongly electron-dispersing nucleoid. This central area is surrounded by a zone consisting of two lamellae with an electron-optically less-dispersing interspace (*8*). The average diameter of these particles is about 1000 A.

Mammary tumors in the mouse may originate in one or more of the hyperplastic nodules that are numerous in the mammary glands of high-tumor-strain mice (*9–12*). The cells composing such hyperplastic nodules must therefore be regarded as potentially precancerous. Are cells of these hyperplastic nodules not showing tumor characteristics, when studied with the light microscope, different from tumor cells in their fine structure, when studied with the electron microscope? Previously we reported that the mitochondria in rat hepatoma showed structural differences from those in liver cells of normal animals. The average diameter of such mitochondria was significantly smaller than that of normal liver mitochondria (*13*). In mammary carcinoma of mice, similar differences in structure and average mitochondrial diameter might be found. This would then constitute a method for making a still finer differentiation between tumor cells and nonmalignant cells, and for the study of acini not yet showing signs of malignant transformation of a tumorous hyperplastic nodule.

Finally, if the formation of these nodules is to some extent dependent upon the effect of the mammary-tumor agent, we might expect accumulations of virus-like particles in hyperplastic mammary-gland tissue of mice. Evidence for the occurrence of virus-like particles in hyperplastic nodules has been previously shown by electron microscopy studies (*14*).

MATERIALS AND METHODS

Animals.—Virgin (♀ WLL × ♂ O2O)F_1 mice with the milk factor were provided by the Antoni van Leeuwenhoekhuis (Netherlands Cancer Institute), Amsterdam. About 80 to 90 percent of spontaneous mammary tumors occur in these mice when they are about 1½ years of age (*15*). Animals used are shown in table 1.

The mice received standard mouse food, wheat grains, and tap water *ad libitum*. They were killed by luxation of the cervical vertebrae.

Tissue preparation.—Tissue samples were taken at random from the tumors and from mammary glands of the same animals, immediately after death. After fixation for 1 hour in an isotonic solution of 1 percent osmic tetroxide (OsO_4) buffered at a *p*H of 7.2 and sucrose added to a final concentration of 4 percent, the tissue blocks were rinsed in distilled water, dehydrated in alcohol, and then impregnated in methacrylate.

For embedding, a mixture of 9 parts butylmethacrylate and 1 part methylmethacrylate was used, to which benzoyl peroxide had been added as a catalyst. Polymerization was done at a temperature of about 45° C.

TABLE 1.—Virgin (♀ WLL × ♂ O2O)F_1 mice and tissue samples

Animal	Age (years)	Weight (g)	Tissue sample
MM1	1½	37½	A) Mammary tumor in right groin: diameter 1½ cm, MM1(T)
Duration of tumor growth about 8 weeks			B) Mammary-gland tissue from second gland on left side, MM1(M)
MM2	1½	37	A) Mammary tumor in right groin: diameter 1½ cm, MM2(T)
Duration of tumor growth about 8 weeks			B) Mammary-gland tissue from second gland on left side, MM2(M)
MM3	1⅔	45½	A) Mammary tumor in right groin: diameter 4 cm, MM3(Ta)
			B) Mammary tumor in right axilla: diameter 2 cm, MM3(Tb)
Duration of tumor growth of MM3(Ta) about 7 weeks			C) Mammary-gland tissue from second gland on left side, MM3(M)
MM4	1⅔	35	A) Mammary tumor in right axilla: diameter 1 cm, MM4(T)
Duration of tumor growth about 3 weeks			B) Mammary-gland tissue from second gland on left side, MM4(M)
MM5	1 11/12	29	A) Mammary tumor in right axilla: diameter 2 to 3 cm, MM5(Ta)
			B) Small tumor nodule in left axilla: diameter 2 mm, MM5(Tb)
Duration of tumor growth of MM5(Ta) about 7 weeks			C) Piece of tissue from transitional area between tumor (B) and surrounding mammary gland MM5(Tc)
MM6	2 1/12	29	Mammary-gland tissue from second gland on right side, MM6(M)
No tumor growth			

Sections of tumor tissue of about 6 μ were made. Parts with a lifelike appearance in accordance with the generally accepted light-microscope criteria were selected. Similar sections were made of the mammary-gland tissue to find the glandular component in the tissue. After selection of those parts which seemed interesting for further study with the electron microscope, the blocks were trimmed. Pyramids were made preferentially within a zone of 100 to 150 μ from the periphery of the tissue block, to make the effect of the fixation as constant as possible.

Ultrathin sections for electron microscopy were made with the glass-knife technique on Porter-Blum, Servall microtomes. The sections were collected on grids covered with a carbon film on Formvar, from which the Formvar had been dissolved with ethylene chloride (*16*). Some of the sections were later stained with lead hydroxide (*17*), and sections were examined with a Siemens microscope (Elmiskop 1).

Montages.—In addition to electron micrographs at higher magnifications, montages covering large areas of tissue sections were made at a magnification of about 11,000. In these montages the topographical relationship of the various tissue components, virus-like particles, the number of such particles, the amount of electron-dense material, and the size and structure of mitochondria could be studied.

Technique for measuring mitochondria.—The diameters of mitochondria as they occurred in the sections were measured by the hundreds. Since measurements were made on all the mitochondria occurring in montages of electron micrographs of large areas of the sections, it seems justifiable to consider this material as a bulk sample of the tissue studied. Measurements were grouped in 0.06 μ and the mean diameter, the estimate of variance, and the standard deviation were calculated by the usual methods (*18*, p. 45). The expected frequencies per class were calculated to establish whether a normal distribution fitted the sample. Furthermore, the agreement between expected frequencies and the actual frequencies was tested with the χ^2 method (*18*, p. 112).

RESULTS

Description of Mammary-Gland Tissue and Tumor Tissue

Light Microscopy

Mammary-gland tissue.—The mammary glands of the old mice were atrophic with hyperplastic areas (fig. 1) different from the inactive glandular tissue of young mice in which no such hyperplastic areas were found. Acinar tubular structures were lying singly or in groups surrounded by stroma consisting of blood vessels, fibrous elements, and fat cells. Some infiltration of the surrounding stroma by lymphocytes and plasma cells was sometimes found along with excretory ducts of varying size. The lining of the acini consisted of a single layer of columnar epithelial cells with round or oval nuclei. The cytoplasm contained secretion droplets and similar droplets were also in the lumen of the acini. Along the periphery of the acini the cytoplasmic extensions of flattened myoepithelial cells were seen.

Tumor tissue.—Tumor tissue consisted of cords and compact masses of polymorphous cells with a high nucleocytoplasmic ratio. Indications of tubule formation were occasionally seen. There were some mitoses, and cords and strands of tumor cells were lying in a poorly developed stroma consisting of blood vessels and fibrous elements. Some macrophages were seen, but secretory phenomena were absent.

At the periphery of a small tumor in one of our animals [MM5(Tc)], transitional forms between acini and tumor tissue could be studied (fig. 2). Since these structures were significant to our problem, special attention was paid to them, as will be seen later.

Electron Microscopy

Mammary-gland tissue.—The hyperplastic areas in the mammary tissue in which no signs of malignant transformation were seen had a uniform appearance in all the animals studied. The epithelial cells lining the acini had round or oval, sometimes polymorphous, nuclei, of which many con-

tained a nucleolus. In addition to fat droplets of varying size, the cytoplasm contained mitochondria and cytoplasmic membrane systems. The former showed the well-known appearance of rod-shaped or ovoid structures with an external double-layered membrane and a certain number of transverse double-layered mitochondrial cristae. The size of the mitochondria will be discussed. The endoplasmic reticulum was less developed than, for instance, the endoplasmic reticulum of liver cells. It consisted of a rather loosely arranged system of membranes enclosing mostly flattened cysternae, which were sometimes distended so that vacuole-like structures were formed. These membranes were externally covered with granules, the so-called particulate matter of Palade (*19*).

The Golgi system consisted of a rather diffuse arrangement of membranes and vesicles, found mostly in the apical portion of the cells. Membranes were not covered with granules as were the membranes of the endoplasmic reticulum (fig. 3).

Epithelial cells showed a villous boundary toward the lumen of the acini. On the basal side of most of these cells an intricate network of stromal fibers was lying directly adjacent to the cell membrane. In other cases, the cytoplasmic extensions of myoepithelial cells separated the epithelial cells from the stromal elements. These myoepithelial cells were easily distinguishable from epithelial cells, since the electron-dispersing capacity of their cytoplasmic ground substance was lower than that of epithelial cells. Furthermore, the cytoplasm of these cells, especially in the cytoplasmic extensions, contained less mitochondria and other cytoplasmic components.

Whereas the boundary against the stroma or the myoepithelial cells was a relatively sharp one, it was not always possible to distinguish a cell membrane between two adjoining cells. Osmiophilic droplets, sometimes lying in electron-optically relatively clear vacuoles and probably belonging to the Golgi system, indicated some sort of secretory activity in the majority of the cells.

Besides such cytoplasmic components as mitochondria and cytoplasmic membrane systems, varying amounts of strongly electron-dispersing material were found in the secretory cells. In some instances this material occurred as aggregates of loose-lying particles of about 70 A. In addition, rounded electron-dispersing bodies with an average diameter of between 0.40 and 0.50 μ were seen in which a strongly electron-dispersing granular matrix was surrounded by a membrane. As figure 4 shows, the loose-lying particles are in some cases distinguishable as a configuration of 4 electron-dispersing dots of about 25 A. In contrast to these particles, other electron-dispersing material occurred as a somewhat amorphous granular material. Text-figure 1, which is a semischematic tracing of a montage of electron micrographs of a section of mammary tissue [MM6(M)], shows how large an amount of strongly electron-dispersing material may occur. In the cytoplasm of the epithelial cells a varying number of virus-like particles was found. These particles were lying singly or in groups in the cytoplasmic substance (fig. 5) and showed the characteristic morphology

of the intracytoplasmic particles described in the literature (*3*). The particles showed a centrally placed, homogeneous area surrounded by a zone of about 140 A consisting of two electron-dispersing layers with a less strongly electron-dispersing interspace.

TEXT-FIGURE 1.—Semischematic tracing of montage of acinar structures of mouse MM6, taken at magnification of 11,000 and reduced to about 1,430. In cytoplasm, the electron-dispersing material, probably consisting of iron-containing pigment, is indicated by black spots.

An average diameter of the particles of about 700 A was in accordance with the value given in the literature (*8*). When virus-like particles were found in cytoplasmic vacuoles or in the lumen of the acini, their morphological appearance differed from that of the intracytoplasmic particles. Such particles had an eccentrically placed electron-dispersing nucleoid lying in a relatively clear, central area which was surrounded by a zone of about 130 A in which two electron-dispersing layers with a less strongly electron-dispersing interspace could be distinguished (fig. 6).

In some instances virus-like particles of the intracytoplasmic type were lying in the top of the villous processes on the cell surface (fig. 7). These villous processes then showed budding as described by Bernhard (*8*). In these cases the location of the virus-like particles seemed to be suggestive of a shift of the particles from the cytoplasm to the lumen of the acini. Some of these particles showed a peripheral zone comprising three layers. Intracytoplasmic virus-like particles were sometimes difficult to distinguish. However, when the sections were stained with lead hydroxide and when high magnifications were used, intracytoplasmic, virus-like particles were found in a great many of the mammary acinar cells of all the animals used.

Virus-like particles were also present in the mammary-gland tissue of an animal which had no tumor [MM6(M), fig. 9]. However, the number of these particles seemed to be slightly lower than in the mammary tissue of animals with a tumor in another gland.

Tumor tissue.—Tumor tissue consisted of cords and strands of polygonal cells surrounded by a poorly developed stroma in which blood vessels and occasional macrophages were seen. In contrast to the majority of the cells which were closely contiguous, some of the tumor cells lay relatively free. A cell membrane was always readily distinguishable, and in most instances was smooth, but occasionally showed villiform processes. This was especially true when the free surface of the cells formed structures faintly resembling acini formations.

Tumor cells showed a strikingly high nucleocytoplasmic ratio. The nuclei sometimes contained a somewhat hypertrophic nucleolus, and occasionally pores could be seen in the nuclear membrane (fig. 10). The cytoplasmic ground substance had a slightly granular appearance; it also contained numerous small vacuoles, possibly of the Golgi system.

Secretory activity was not evident. Besides a few fat vacuoles, the cytoplasm contained mitochondria and a poorly developed endoplasmic reticulum. Most of the mitochondria had an abnormal aspect: While they showed a bilaminar outer boundary, the mitochondrial cristae which are readily discernible in normal cells were infrequent or even entirely absent (fig. 11). The general impression was that the diameter of the mitochondria was somewhat lower than that of mitochondria in mammary-gland tissue which did not show any signs of malignant transformation. This point will be discussed in more detail later.

Besides a poorly developed endoplasmic reticulum, a rather highly developed Golgi system was found. In normal cells this system was located in those parts facing the lumen of the acini, but this was not true in the tumor cells in which the Golgi system consisted of a rather widely dispersed system of membranes and vesicles (fig. 11). As a result of the relative hypertrophy of this system in many of the tumor cells, it gave an impression of some activity. This was in contrast to findings for the other cytoplasmic components definitely underdeveloped.

Strongly electron-dispersing elements such as were found in the cells of mammary-gland tissue and which seemed to consist of deposits of iron-containing material were lacking in the majority of the tumor cells.

Deposits of this kind were in a few cells of a small tumor [MM5(Tb)]. In a great number of cells, however, strongly electron-dispersing material was found which showed evidence of osmiophilia rather than a possible intrinsic electron-dispersing capacity, as with ferritin particles. This electron-dispersing material consisted of multilaminar structures plus an amorphous strongly electron-dispersing material (fig. 12). The spacing of the laminae was somewhat variable: In most of the formations it was about 45 A, in a few others about 120 A, and in some instances transitions were seen from the 45 A type of spacing to the 120 A type (fig. 13). With respect to the spacing, these formations agree well with those found in similar structures in degenerating liver tissue (Daems, personal communication). Structures of this kind showed some resemblance to the formations described as myelin figures by Policard *et al.* (*20*) and Stoeckenius (*21*). They seemed to consist of phospholipides and we can possibly regard them as cell-degeneration products. Formations of this kind were in the cytoplasm of tumor cells and of stromal macrophages.

One of the most striking findings in tumor tissue was the relatively low number of virus-like particles, as compared with that in the mammary-gland cells of the same animals. Even when the sections were stained with lead hydroxide to increase the contrast, aggregates of virus-like particles were found only in relatively few cells, whereas the majority of the cells contained none. Most of the particles were of the intracytoplasmic type. Particles of the extracellular type were occasionally encountered in the tissue. In some instances virus-like particles were lying in a vacuole, the wall of which was covered with Palade granules and therefore seemed to form part of the endoplasmic reticulum (fig. 8).

In one of the animals (MM5), which had a large mammary-gland tumor on the right side, a small tumor with a diameter of 2 mm was observed in the left axilla. The large tumor showed the cell characteristics described previously. The tumor on the left side, however, showed aspects which were of special interest and warrant further discussion here.

Transitional forms between mammary-gland tissue and tumor tissue.— The small tumor in the left axilla of one animal (MM5) seemed on macroscopic examination to be a hyperplastic nodule. It was grayish-white and had a somewhat friable consistency. Light-microscope examination, however, revealed a tumor formation within the nodule. In the peripheral region of the tumor, transitional forms between the mammary-gland tissue and tumor tissue were seen (fig. 2). The mammary-gland tissue showed the formation of acini with a normal histologic pattern in which secretory activity was present. More solid epithelial formations were lying intermingled with the acinar formations. Although some fat droplets were found in the solid elements, there appeared to be no secretory activity. Tissue blocks from the center of the nodule consisted solely of tumor tissue. It seemed of interest to study the fine structure of the borderline acini. Electron-microscope examination of these revealed a certain degree of thinning of the epithelial lining, evidently as a result of an excessive desquamation of the superficial portions of the cells. Villiform processes

on the cell surface were rare. Secretory activity was still present, as evidenced by the large number of osmiophilic droplets of varying size in both the cytoplasm of the cells and in the lumen of the acini. In general, the composition of the cells with regard to the nucleocytoplasmic ratio and the structure of the mitochondria and of other cytoplasmic components did not seem to be greatly altered, with the exception of the electron-dispersing material and the virus-like particles. Ferritin particles and other iron-containing pigment with a probable intrinsic electron-dispersing capacity were less frequent, while some multilaminar electron-dispersing structures were occasionally seen which could be interpreted as myelin figures.

The most striking feature, however, was the large number of virus-like particles, practically all of the extracellular type, found in both the lumina of the acini and in vacuoles in the cytoplasm of the acinar cells (figs. 14 and 15). Vacuoles containing these particles were sometimes covered by only a narrow rim of cytoplasm, bulging into the lumen of the acinus. Parts of the cells had desquamated, and in such instances we were unable to find virus-like particles in the tissue blocks from the center of the tumor, nor did the majority of the tumor cells contain deposits of iron pigment. Electron-dispersing material of this kind was in only a few cells.

Summarizing, we may say that we are concerned with acinar formations in the periphery of a small tumor. The acini showed no signs of malignant transformation under light-microscope examination. Electron-microscope examination revealed an excessively high number of virus-like particles absent in structures that could conclusively be recognized as tumor elements. These borderline acini showed a definite loss of electron-dispersing material in comparison with the mammary-gland tissue from other animals. Furthermore, signs of cell degeneration were present.

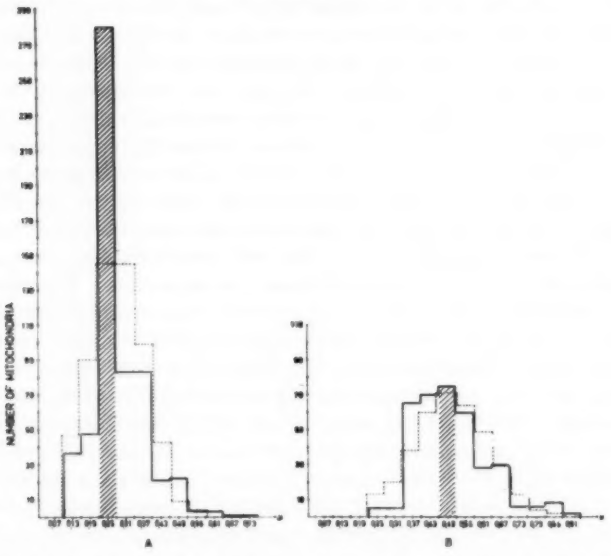
The question now arises whether we have still another criterion for establishing that the cells composing the borderline acini should be regarded as cells with or without malignant transformation. In the following section, we will report the results of our study of the average diameter of the mitochondria in various kinds of tumor tissue and mammary-gland tissue without signs of malignant transformation.

Size of Mitochondria in Mammary-Gland Tissue and Mammary Tumors

The diameter of the mitochondria in mammary-tumor tissue from several mice was measured in the montages of electron micrographs covering large areas of the sections. Since all mitochondria in several acini were studied, these measurements can be regarded as a bulk sample of the tissue. The value of the average diameter, the estimate of variance, and the standard deviation ("s") were calculated from these measurements. The total number of mitochondria measured in each case is indicated as "n."

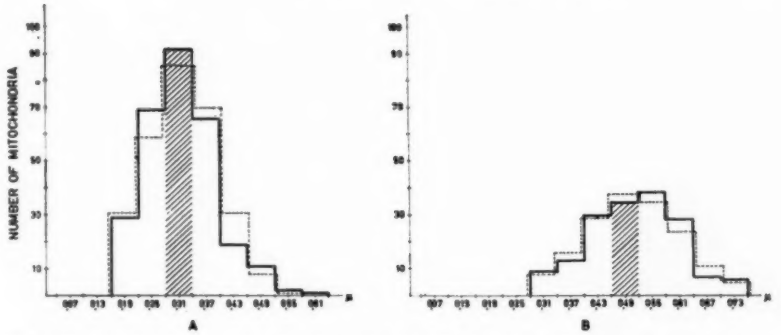
The values for the average diameters in the tumor tissue of MM2(T),

MM3(Ta), and MM5(Tb) were 0.28 μ (s = 0.08 μ; n = 581), 0.31 μ (s = 0.08 μ; n = 289), and 0.32 μ (s = 0.09 μ; n = 751), respectively (text-figs. 2A, 3A, and 4A). As will be seen, there is only a slight difference between the values for the average diameters in the various samples. These values were compared with those for the average diameters of the mitochondria in mammary-gland tissue of the same animals which showed no signs of malignant transformation [MM2(M), MM3(M), and MM5 (Tc)]. It must be kept in mind that the last of these specimens consisted of mammary-gland tissue without signs of malignant transformation surrounding the tumor MM5(Tb). Furthermore, mammary-gland tissue of an animal with no tumor was examined [MM6(M)].

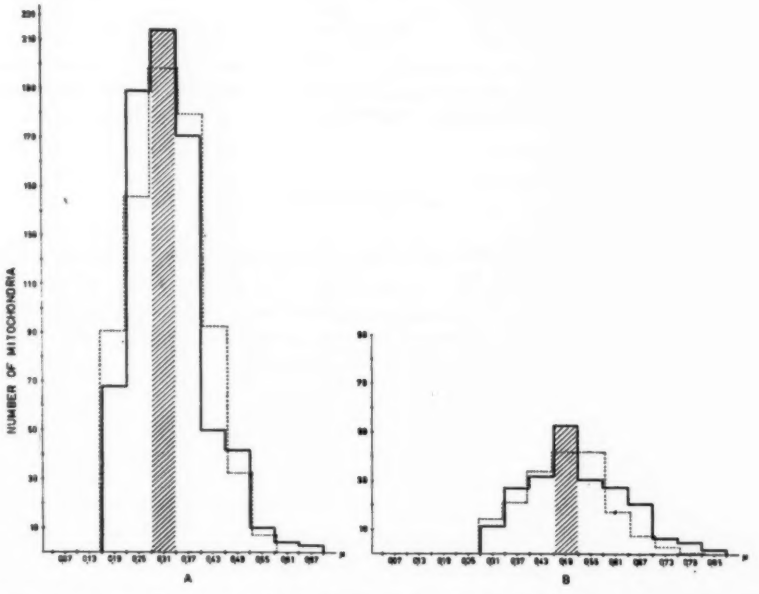


TEXT-FIGURE 2.—Frequency distribution of mitochondrial diameters in mammary-tumor tissue (A) and mammary-gland tissue without signs of malignant transformation (B) in mouse MM2. *Drawn* lines: actual frequencies; *dotted* lines: expected frequencies.

The average diameter of the mitochondria in MM2(M), MM3(M), MM5(Tc), and MM6(M) was 0.50 μ (s = 0.12 μ; n = 361), 0.51 μ (s = 0.10 μ; n = 161), 0.52 μ (s = 0.12 μ; n = 217), and 0.52 μ (s = 0.11 μ; n = 360), respectively (text-figs. 2B, 3B, 4B, and 5). It is clear that in this material, as well as in tumor tissue, the values of the average diameter of the mitochondria show a remarkable constancy. It is also clear that in mammary tumors a constantly lower value is found for the average mitochondrial diameter when compared with that of the mammary tissue without signs of malignant transformation. This is of special interest with respect to the findings in mouse MM5: Acini in the border area

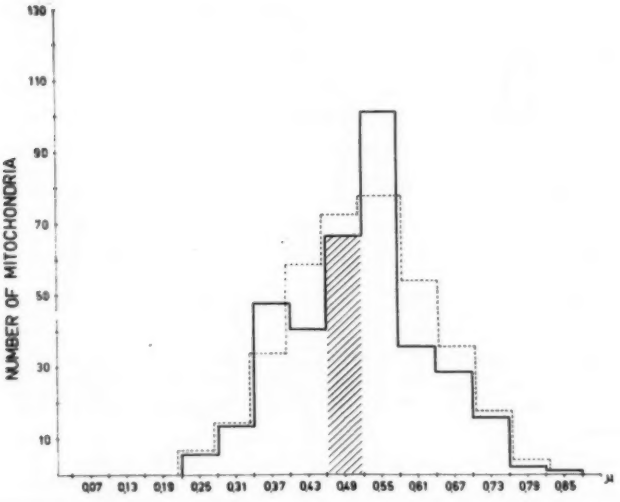


TEXT-FIGURE 3.—Frequency distributions of mitochondrial diameters in mammary-tumor tissue (A) and mammary-gland tissue without signs of malignant transformation (B) in mouse MM5. *Drawn* lines: actual frequencies; *dotted* lines: expected frequencies.



TEXT-FIGURE 4.—Frequency distributions of mitochondrial diameters in mammary-tumor tissue (A) and mammary-gland tissue without signs of malignant transformation (B) in mouse MM5. *Drawn* lines: actual frequencies; *dotted* lines: expected frequencies.

between tumor tissue and the surrounding mammary gland, which in the light microscope did not show a clear malignant transformation but which in the electron microscope evidenced cell degeneration and an extremely high number of virus-like particles, did not show signs of malignant transformation with respect to the average diameter of the mitochondria.



TEXT-FIGURE 5.—Frequency distributions of mitochondrial diameters in mammary-gland tissue of a mouse in which no tumor was found (MM6). *Drawn* lines: actual frequencies; *dotted* lines: expected frequencies.

DISCUSSION

Nodules consisting of local hyperplasia of acini in the mammary glands of mice were described by Haaland in 1911 (*22*). Since then many reports have dealt with the character of these nodules and their significance as lesions in which mammary cancer can originate (*9–12, 23–25*).

In the earlier work the presence of hyperplastic nodules in agent-free mice was rarely observed (*26*), whereas in animals with the agent relatively large numbers of nodules were seen. Later work established the presence of nodules in mice of various agent-free strains, though in smaller numbers and in older animals (*23, 24*). It was shown that the number of animals with nodules and the number per animal increased from virgins to breeders and from breeders to forced-breeders (*2, 24*). This and other work (*27, 28*) indicated that the development of hyperplastic nodules is hormone-dependent.

Besides this hormone dependency, the development of hyperplastic nodules and mammary tumors depends upon the genetic constitution, the presence of the mammary-tumor agent, and environmental factors. The mammary-tumor agent seems to act as an accelerator on the process of nodule formation, the transition of nodules into tumors being dependent on the genetic constitution (*24, 29*).

The presence of virus-like particles visible with the electron microscope and probably related to the mammary-tumor agent was described in mammary-tumor tissue (*7, 8*) and also in hyperplastic nodules by Pitelka

et al. (*14*). The latter authors considered the presence of the virus-like particles in hyperplastic nodules as a means of differentiating these structures on the fine structural level from active, prelactating, normal mammary tissue in which practically no virus-like particles, clearly recognizable as such, were found.

In our material we found virus-like particles in the hyperplastic mammary tissue of old mice, sometimes in fairly large numbers. This agrees well with the assumption that mammary-tumor agent is present in the mammary-gland tissue before malignant transformation occurs and that it can therefore have some influence on the development of precancerous hyperplastic nodules.

One striking feature was the great number of particles, nearly all of them of the extracellular type, in the borderline acini of a small tumor, whereas in the tumor component itself practically no particles were seen, which was also true to some extent in the other tumors studied. These borderline acini probably belonged to the pre-existing hyperplastic tissue in which the tumor had developed. With respect to this point it was important to determine whether these acini showed signs of malignant transformation. In the light microscope these individual acini could be distinguished from the tumor components. With the electron microscope we found another criterion by which the acini could be distinguished from tumor tissue: In the mammary-gland tissue of the animals studied, which in the light microscope showed no signs of malignant transformation, we found an average mitochondrial diameter of about 0.50 μ. In the tumor tissue of the same animals, an average mitochondrial diameter of about 0.30 μ was found. This showed some resemblance to the results of rat hepatomas in which a similar difference was found between the average diameter of mitochondria of the hepatoma and the average mitochondrial diameter of normal hepatic cells (*13*). Whereas the average mitochondrial diameter in liver tumors, induced with *N,N*-dimethyl-*p*-phenylazoaniline plus rice diet, and the average mitochondrial diameter in liver cells surrounding the tumor were about the same, this did not occur in mammary-gland tissue.

A difference was also found between the average mitochondrial diameter of a small mammary tumor and the average mitochondrial diameter of its borderline acini which clearly belonged to the hyperplastic nodule in which the tumor had developed. It was clear, therefore, that the borderline acini did not resemble tumor tissue, but that these acini bore a general resemblance to hyperplastic mammary tissue without malignant transformation. The cells of these borderline acini were flat and showed some signs of degeneration. Myelin figures were sometimes present, while the iron content was low. Large vacuoles containing numerous virus-like particles and a few fat droplets were visible in the cytoplasm; in the lumen cytoplasmic fragments were seen. Particles of the extracellular type were found both in the lumina and in the cytoplasmic vacuoles. In these acini there was no indication of a transition at the cell membrane of intracytoplasmic particles to those of the extracellular type, as with nondegenerat-

ing cells. Since desquamated cytoplasmic fragments containing vacuoles were seen in the lumen of the acini, it seems probable that the great number of particles is a result of degeneration of the epithelial cells. It is likely that the degeneration in these cells should be regarded as a result of tissue disturbances provoked by tumor growth nearby.

In our material a large amount of electron-dense material, probably iron pigment and consisting partly of ferritin, was seen in mammary-gland tissue without evidence of malignant transformation. This pigment was absent in most of the tumor cells studied. These last findings are in close agreement with the results reported in the literature (*30*, *31*).

ADDENDUM

Since virus-like particles were found in very large numbers, especially in the hyperplastic mammary-gland tissue of mice with the milk factor, it was of interest to study the hyperplastic tissue of mice without the milk factor. Recently we investigated, with the aid of the electron microscope, hyperplastic tissue of 2 C3Hf breeder mice at the age of about 1½ years, which were provided by Dr. O. Mühlbock and Dr. L. M. Boot from the Antoni van Leeuwenhoekhuis (Netherlands Cancer Institute), Amsterdam. Breeder mice belonging to this substrain have a mammary-tumor incidence of 44 percent at a mean age of 563 days. One of these mice that had had 7 litters was killed at the age of 18 months. It belonged to a substrain that was foster nursed for 28 generations. The other mouse that had had 2 litters was killed at the age of 19 months. This mouse belonged to a substrain foster nursed for 29 generations. In these mice also, large numbers of virus-like particles were found in the hyperplastic tissue components. Figures 16 and 17 show the intracytoplasmic and extracellular type of particles occurring in this kind of tissue. Morphologically they are identical with the particles found in hyperplastic tissue and tumor tissue of mice with the milk agent.

We may conclude, therefore, that large numbers of virus-like particles are also present in hyperplastic mammary tissue of mice of a high-mammary-cancer strain, freed from the milk-factor by foster nursing. This finding is in close agreement with the results reported by Pitelka *et al.* (*32*). The relation between the mammary-tumor agent and the visible virus-like particles is therefore not clear. Further work on this subject is in progress.

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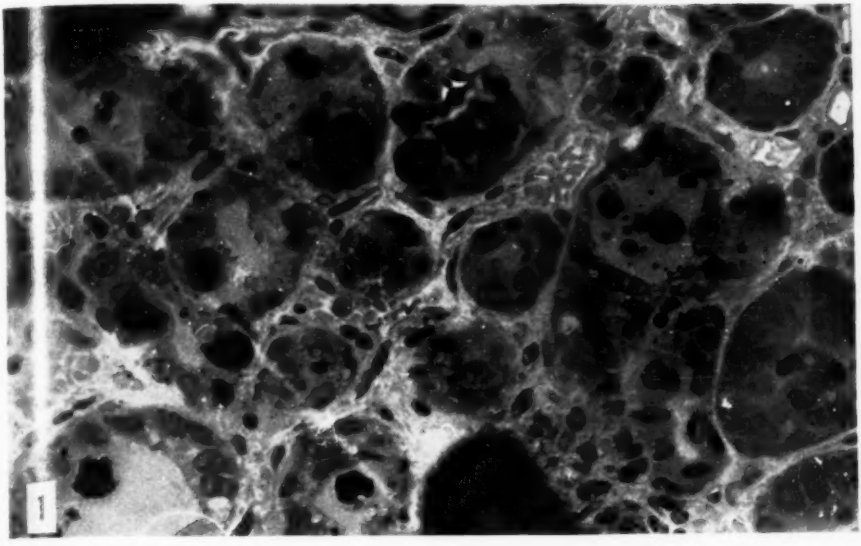
PLATE 118

FIGURE 1.—Light-microscope view of mammary-gland tissue of mouse MM3 showing no signs of malignant transformation. Glandular hyperplasia. Osmic tetroxide fixation. Hematoxylin. × 508

FIGURE 2.—Light-microscope view of hyperplastic mammary-gland tissue on *left* and tumor tissue on *right* (mouse MM5). Acini within outlined square showing no signs of malignant transformation were selected for electron microscopy. Osmic tetroxide fixation. Hematoxylin. × 205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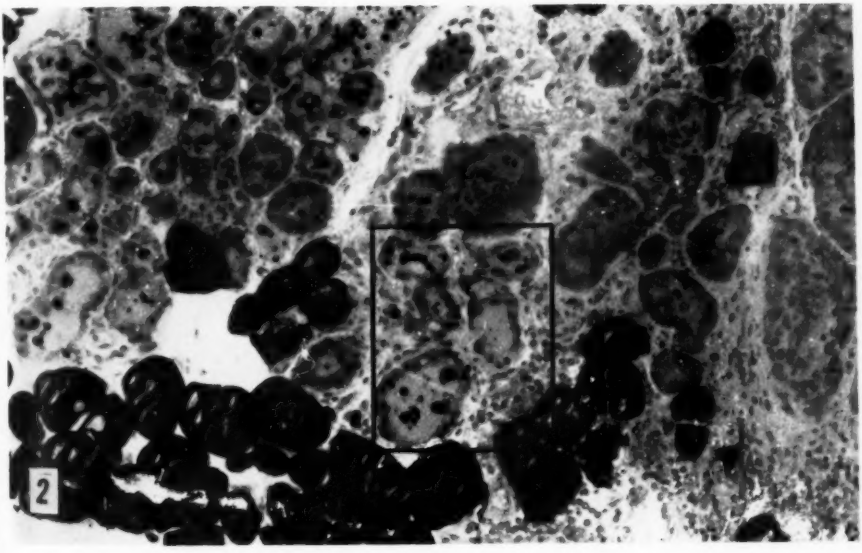
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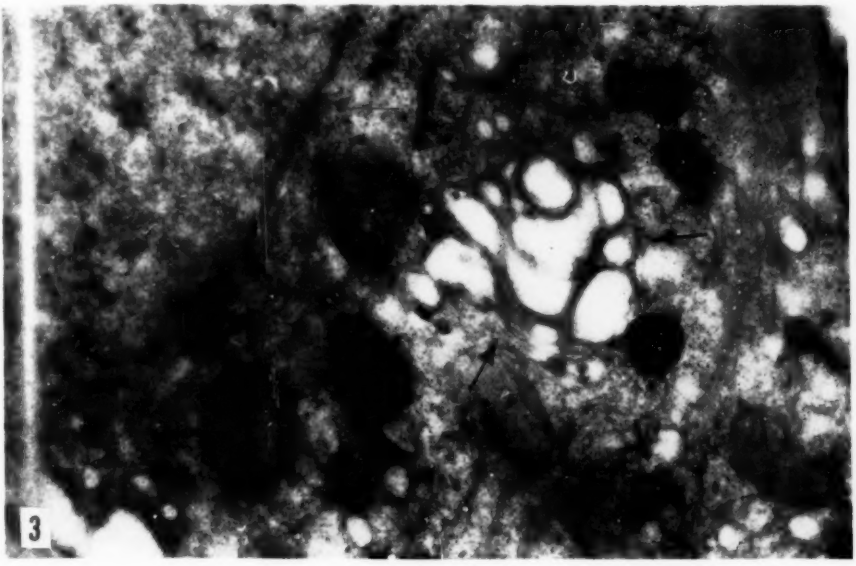
PLATE 119

FIGURE 3.—Portion of acinar cell in mammary tissue without signs of malignant transformation (mouse MM3) showing on *left* a part of nucleus and well-developed Golgi system in the cytoplasm (*arrows*). About × 36,000

FIGURE 4.—Part of acinar cell in mammary tissue without signs of malignant transformation (mouse MM5) showing electron-dense bodies consisting of granular material surrounded by a membrane which is clearly bilaminar in some corpuscles. In cytoplasm between corpuscles numerous electron-dispersing particles can be found. About × 92,000
Insert: Higher magnification showing small particles in which pattern suggestive of ferritin can be seen. About × 250,000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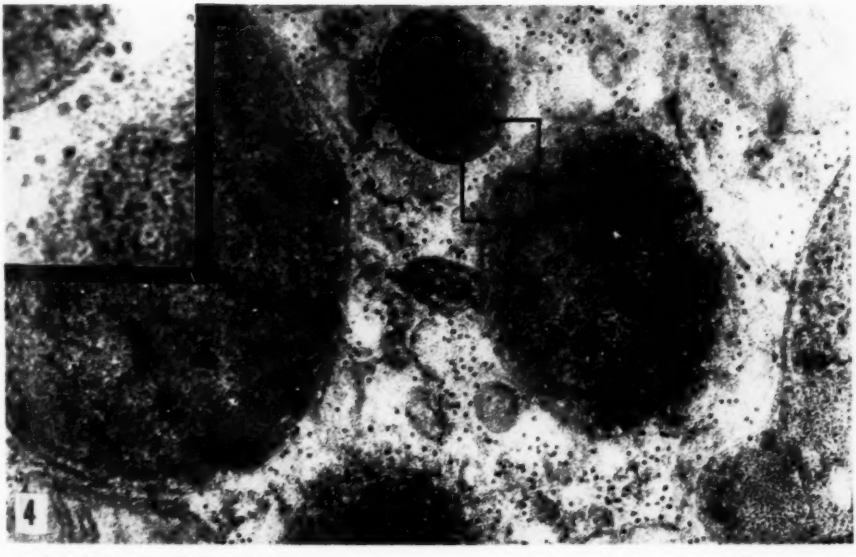
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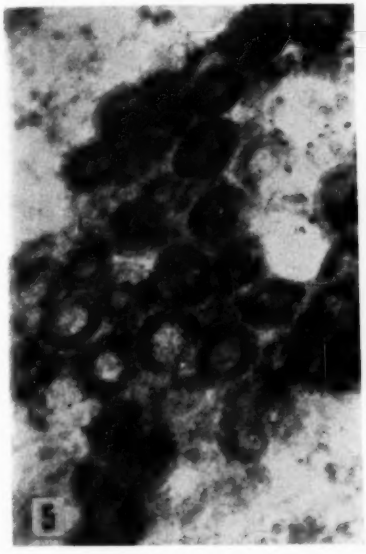
PLATE 120

FIGURE 5.—Group of intracytoplasmic, virus-like particles in a tumor cell of mouse MM4. Lead hydroxide staining. About × 107,000

FIGURE 6.—Group of extracellular, virus-like particles in lumen of acinus of mouse MM5 showing an eccentrically placed nucleoid. Lead hydroxide staining. About × 107,000

FIGURE 7.—Part of cytoplasm of acinar cell in mammary tissue not showing signs of malignant transformation (mouse MM5). Numerous intracytoplasmic particles are visible. At *upper right* is configuration of particles suggestive of transition of particles from cytoplasm to lumen. Lead hydroxide staining. About × 80,000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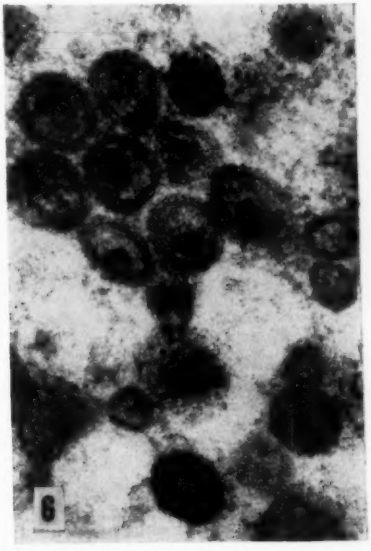
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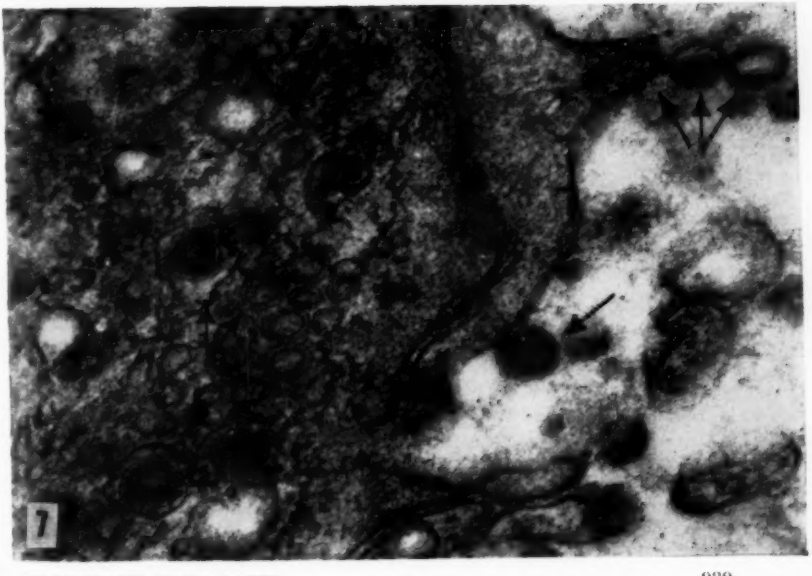
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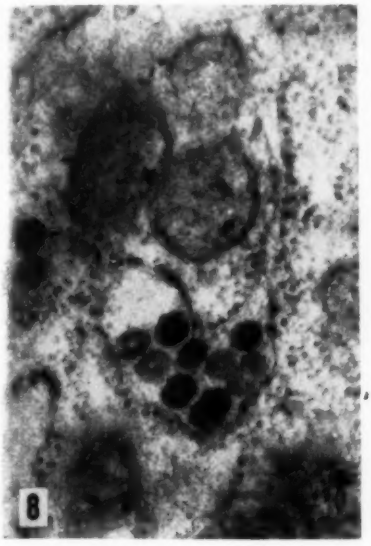
PLATE 121

FIGURE 8.—Virus-like particles lying in distended cistern which seems to be covered with Palade granules. Lead hydroxide staining. About × 60,000

FIGURE 9.—Intracytoplasmic particles occurring in acinar cell of hyperplastic mammary tissue of mouse MM6 in which no tumor was found. Lead hydroxide staining. About × 120,000

FIGURE 10.—Part of cytoplasm and nucleus of mammary-tumor cell of mouse MM3. Pores are visible in nuclear membrane (*arrows*). About × 60,000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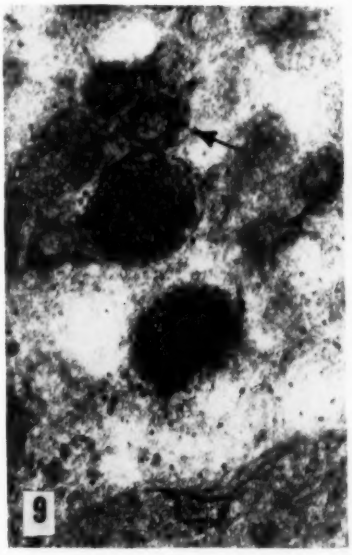
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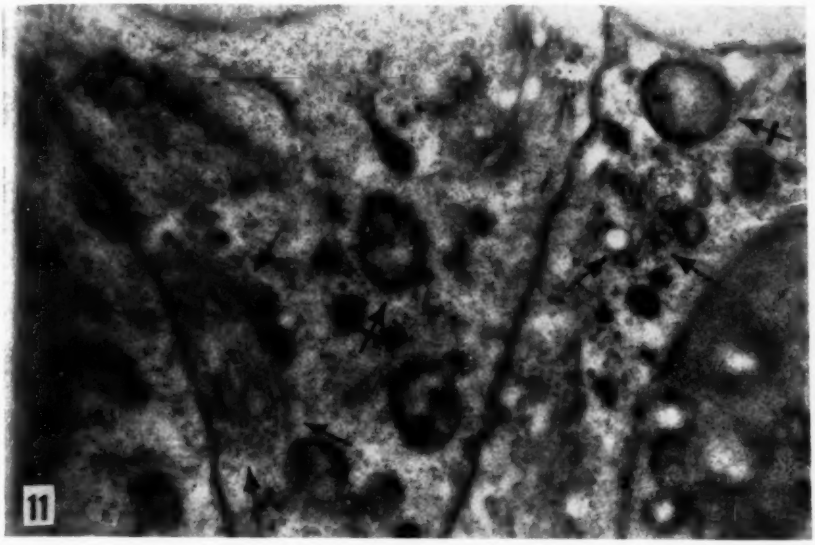
PLATE 122

FIGURE 11.—Part of cytoplasm and nuclei of 3 adjoining tumor cells of mouse MM3. A highly developed Golgi system is seen (→). Mitochondria (⊣→) show loss of cristae mitochondriales. About × 42,000

FIGURE 12.—Myelin figures showing regular spacing of about 45 A in tumor cell of mouse MM5. About × 115,000

FIGURE 13.—Myelin figures in tumor cell (mouse MM5) showing transition from regular 45 A spacing in *lower* area to wider spacing in *upper part* of photograph. About × 68,000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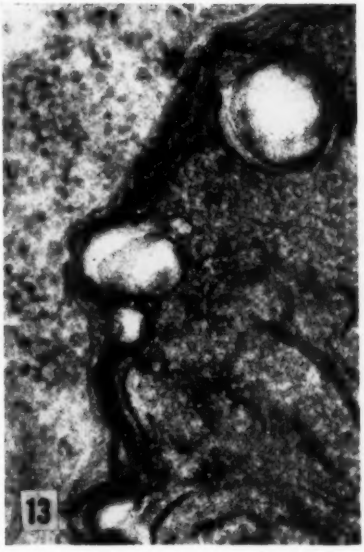
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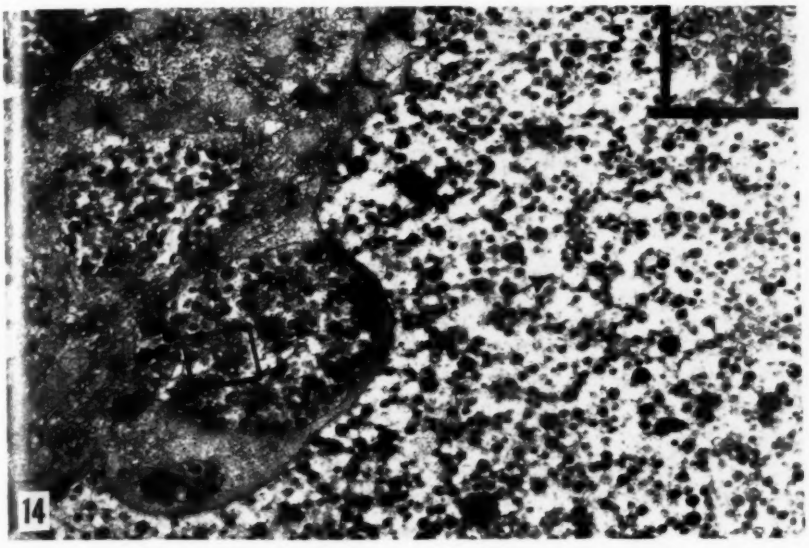
PLATE 123

FIGURES 14 AND 15.—Part of cytoplasm of cells lining acinus showing no signs of malignant transformation in borderline area of small tumor in mouse MM5. Large numbers of virus-like particles are visible both in lumen and in cytoplasmic vacuoles. Part of cytoplasm containing such vacuoles sometimes protrudes into lumen. Apart from virus-like particles, a certain number of osmiophilic particles showing no internal structure, probably secretion droplets, may be seen in the pictures. Lead hydroxide staining. × 11,500
Insert. × 25,000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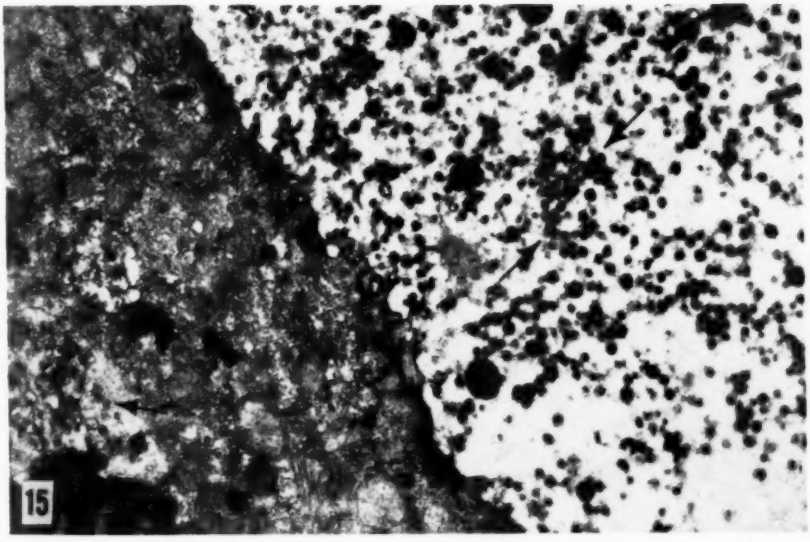
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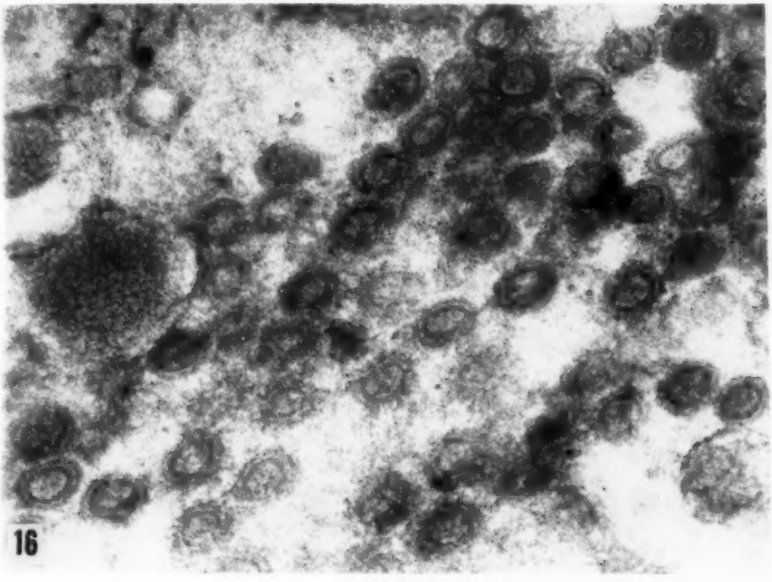
PLATE 124

FIGURE 16.—Virus-like particles of intracytoplasmic type in hyperplastic tissue of C3Hf mice. Lead hydroxide staining. × 112,000

FIGURE 17.—Virus-like particles of extracellular type in hyperplastic tissue of C3Hf mouse. Lead hydroxide staining. × 112,000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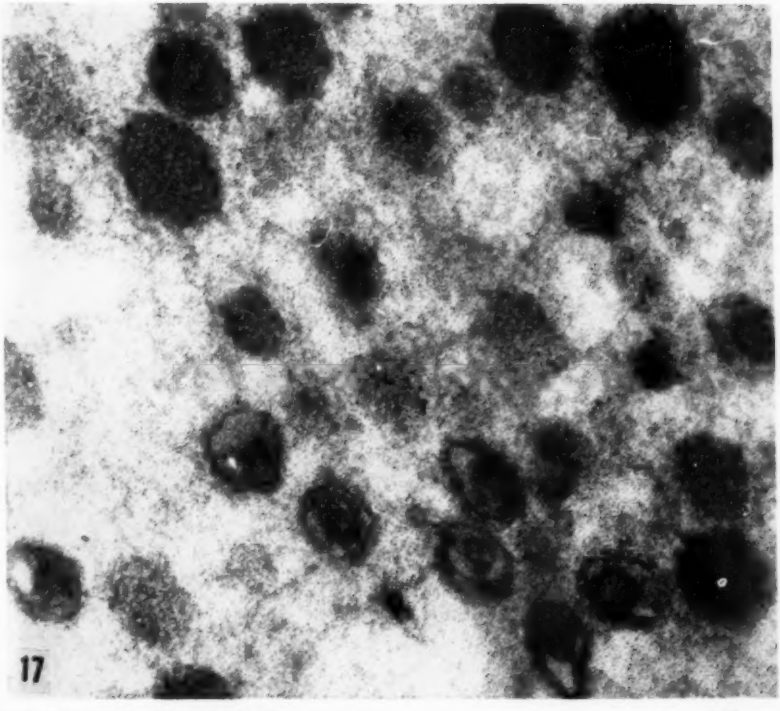
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A Contagious Tumor of the Hamster [1]

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SUMMARY

A spontaneous reticulum-cell sarcoma of the hamster was successfully carried through 12 passages. Generalized cutaneous involvement was present in the first and second passages. Thereafter, the growth was typified by widespread metastases and a large, ulcerating tumor at the injection site, with local skin invasion only. In the terminal phases of transplanted growth, the peripheral blood became flooded with tumor cells. Injections of blood, ascitic fluid, and brain from tumor-bearers produced tumor in the host. Cell-free filtrates of brain, ascitic fluid, and tumor have not produced tumor. Ten untreated animals were caged with 10 animals inoculated with the tumor. Of the untreated animals, 7 died with laryngeal obstruction, of which 6 had submucosal tumor growth primary in the larynx, and 1, a mass in the posterior pharynx. Two animals, with the generalized systemic involvement similar to that caused by solid tumor inoculations and without laryngeal involvement, were killed. Three uninoculated animals were separated by a screen from 3 inoculated animals; one of the uninoculated group developed tumor in the larynx. Fresh tumor tissue was fed to 6 separated animals. One developed a systemic tumor involvement, another the laryngeal obstruction only.—J. Nat. Cancer Inst. 26: 949–957, 1961.

THERE HAVE been reports of three animal tumors which are contagious. Cellular transfer is essential for the contagiousness of one, the venereal tumor of the dog (*1*, *2*). The others, a visceral lymphomatosis of the chicken and the Rous sarcoma, have viruses as known causative agents (*3*, *4*). We have observed a fourth contagious neoplasm which arose in an untreated laboratory hamster.

The original tumor appeared spontaneously in the upper lip of a 732-day-old male hamster. It was firm, 18 × 18 × 5 mm, and white on the cut surface. Histologically it was made up of closely packed mononuclear cells with relatively clear macrophages scattered throughout. It was diagnosed as a reticulum-cell sarcoma (figs. 1 and 2). There were no metastases.

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[1] Received for publication October 21, 1960.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

METHODS AND RESULTS

Solid Tumor Transplants

Subcutaneous injections were made by trocar into the right flank, of pieces approximately 1 mm square of freshly excised tumor tissue. Male golden hamsters (*Mesocricetus auratus*) randomly bred at the National Institutes of Health colony were used in all procedures, and except for the first- and second-transplant generations the ages ranged from 1 to 2 months. The 6 animals receiving the first-generation transplants were 223 days old; the 16 animals receiving the second-generation transplants ranged from 93 to 294 days of age. A total of 159 hamsters, representing 12 transplant generations, received subcutaneous inoculations. The animals were kept until they appeared moribund and were then killed with chloroform. Blood was drawn by a capillary pipette from the venous plexus behind the eye. The blood smears were stained by Wright's method (*5*).

In the first-transplant generation, all animals showed a widespread tumor infiltration of the skin with ulcerations. There was no localized tumor at the injection site. The lymph nodes, liver, spleen, and kidneys were enlarged; microscopically they were infiltrated with tumor. No nodular tumor formations were present in these organs.

In the second-transplant generation 2 animals had similar widespread infiltration of the skin. The remaining 14 animals of this generation and all of the 10 subsequent generations developed discrete localized tumor growths at the injection site (fig. 3). Only the skin over the tumor was infiltrated with loss of hair, central ulceration, and scab formation. The tumor on section was firm and white. Occasionally strands of yellow, necrotic material were found directly beneath the scab. There was tumor extension to the regional lymph nodes and to distant lymph nodes, both visceral and peripheral. The liver and spleen, especially in later transplants, were greatly enlarged by tumor infiltration. The kidneys were enlarged, pale, and splotched with hemorrhage. Histologically the kidneys showed extensive interstitial infiltration. The liver invasion was at first predominantly portal, but in later generations the sinusoids were also involved. With extensive peritoneal tumor growth, ascites was generally present. Except for 4 animals of the second-transplant generation, which had tumor infiltration of the esophagus and forestomach, there were no metastases to the digestive tract.

In the transplant series as a whole there were widespread tumor metastases to the pancreas, salivary glands, heart, lungs, mesentery, seminal vesicles, epididymis, and bone marrow, though each animal did not necessarily have infiltration of all these organs. Neither the brain nor spinal cord contained tumor, though the meninges of the spinal canal were occasionally infiltrated.

In the terminal phases the peripheral blood was flooded with tumor cells which comprised from 30 to 70 percent of the total white count. The cells resembling monocytes were rounded (fig. 4), usually contained

a nucleolus and a few azurophilic granules, and were peroxidase-negative.

The survival time, which in the first-transplant generation ranged from 86 to 257 days post inoculation, decreased to approximately 30 days for the latest generations. The age of the host after weaning did not affect this latter survival time.

Blood and Ascites Injections

Five animals were inoculated intraperitoneally with 0.5 cc whole blood from a moribund tumor-bearing animal with ascites, and 5 animals intraperitoneally with 1 cc of the ascitic fluid. All these injections resulted in generalized tumor growth in the peritoneum with invasion of most abdominal organs.

Intratracheal Inoculations

Three hamsters were inoculated intratracheally with 0.1 cc ascitic fluid from a tumor-bearing animal. The trachea was first exposed through a neck opening, and the injection needle inserted through the wall into the tracheal lumen near the larynx. The first animal was killed after 4 days, and the next 2 were killed after 7 days, all with symptoms indicating laryngeal obstruction. In all cases, microscopic examination revealed tumor thickening of the laryngeal mucosa and infiltration of the pericartilage tissue. There were no metastases.

Brain Tissue Transplants

Four animals were inoculated subcutaneously with small pieces of brain from a moribund tumor-bearer. These inoculations produced tumors indistinguishable from those produced by solid tumor implants.

Transmission

Contact exposure.—Ten intact hamsters were caged with ten others having subcutaneous inoculations of the tumor. When an inoculated animal died, it was replaced by one freshly inoculated. After 31 days one uninoculated animal developed the unusual symptoms of wheezing, dyspnea, and cyanosis leading rapidly to death. The only gross lesion was a marked thickening of the laryngeal mucosa. Microscopically this this was the result of extensive tumor infiltration into the lamina propria (figs. 5 and 6). The epithelium remained intact and neither the trachea nor the pharynx was involved. Blood smears were negative for tumor cells.

Within 10 months after the exposure began, a total of 7 animals died at 31, 43, 48, 62, 124 (2 animals), and 301 days with identical symptoms of respiratory obstruction. In 4 the tumor was confined to the laryngeal mucosa; in 1 there were small infiltrates to a regional lymph node, and in another the tumor extended beyond the laryngeal cartilage into the

muscular wall of the esophagus. In still another there was tumor growth in the posterior pharynx, with infiltration of the cervical lymph nodes. There was no attempt to transplant the laryngeal tumors.

In addition to these 7 animals, 2 of the 10 exposed were killed because of widespread tumor metastases similar to those that had received transplants of solid tumor. The larynges of these 2 were not affected. Tumor tissue from both was successfully transplanted subcutaneously. In none of the infected animals was there evidence of digestive-tract involvement. One of the 10 exposed was destroyed by cannibalism.

Limited contact exposure.—Three inoculated and 3 uninoculated hamsters were placed in the same cage, the 2 groups separated by a permanent wire screen of ¼-inch mesh. After 208 days 1 uninoculated animal died of tumor obstructing the larynx. The remaining 2 uninoculated animals were healthy after 245 days.

Tumor feeding.—Five separately caged animals were fed fresh tumor tissue twice a week. Within 128 days two had developed tumors. One of these was killed at 100 days because of an enlargement in the neck. At autopsy the cervical lymph nodes were tumorous, and there were generalized metastases similar to those produced by solid tumor inoculations (fig. 7). The second hamster became dyspneic and died at 128 days; only the larynx was involved. In neither animal was the digestive tract affected.

Filtrate injections.—Cell-free filtrates of solid tumor tissue, ascitic fluid, and brain from tumor-bearing animals were inoculated intraperitoneally, intratracheally, and subcutaneously into weanling hamsters. None of the filtrates have produced tumor growth.

DISCUSSION

The generalized cutaneous involvement in hamsters bearing the first tumor-transplant generation was unique and was reminiscent of the human disease mycosis fungoides. However, it did not reappear after the second-transplant generation. In subsequent transplant generations the disease process became uniform for all inoculated animals. The widely infiltrating tumor metastases seemed to follow the lymphatics or vascular adventitia, forming no discrete lesions except at the injection site. Although microscopically tumor was not found in the brain of moribund tumor-bearers, there were tumor cells abundant in the circulating blood. This probably accounts for the success of the brain transplants.

This tumor is unlike those previously described for hamsters, with two possible exceptions. Ashbel, in 1945 (*6*), reported the successful transmission of a "polymorph sarcoma" by intraperitoneal inoculation of blood from the diseased animals. No blood examination was reported and attempts to transmit it by filtrates of tumor and blood unsuccessful. Recently Greene and Harvey reported a spontaneous transplantable lymphoma of the hamster with early release of tumor cells into the cir-

culating blood (*7*). We have found no other reports of leukemia, or leukemia-like diseases in hamsters, either induced or spontaneous.

The contact-exposure experiments have shown that uninoculated hamsters caged with tumor-bearing hamsters will develop the tumor. In addition, the limited contact-exposure experiments have shown that the disease can be transmitted even when the hamsters are separated by a screen. Since the separation was only a single thickness of wire mesh, cellular material could be transmitted from the inoculated to the uninoculated animals.

It has been our observation that hamsters are cannibalistic, and will devour a dead cage mate. They will gnaw at an open wound or ulceration on themselves or others. For this reason, the tumor was fed to separated animals with the aforementioned positive results. Maduros *et al.* (*8*) have recently reported their success in the oral induction of leukemia in mice by brain mince and brain filtrate. Also, Schmidt (*9*) has reported the transmission of Walker carcinoma, Jensen sarcoma, and Yoshida sarcoma in rats, and Sarcoma 37 and DMBA spindle-cell sarcoma in mice by feeding the respective tumor tissue. His results were variable, with a high percentage of tumor growth arising in the ingestive and digestive tract and often in the lungs.

In the exposure experiments and in the tumor-feeding experiments there were two growth patterns: one of generalized systemic tumor involvement and the other of isolated laryngeal involvement with obstruction, neither one occurring in the same animal. Whether these patterns represent two distinct manifestations of a single mechanism, or whether they represent entirely different processes of tumor transfer and spread, has remained unanswered. It is apparent that cellular transmission is involved in this contagiousness, but this does not exclude the possibility that the disease may be induced by a subcellular etiologic agent. All attempts to reproduce the tumor through cell-free filtrates of tumor tissue have been unsuccessful to date.

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PLATES

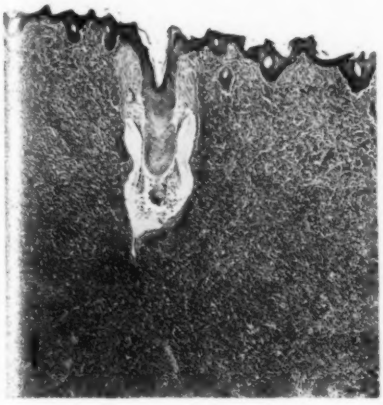
PLATE 125

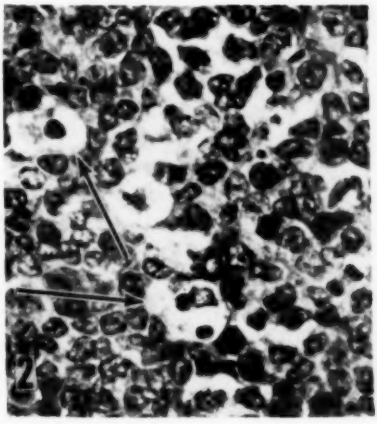
FIGURE 1.—Original spontaneous sarcoma, composed of closely packed cells in the dermis. Neither epidermis nor hair follicle invaded. × 37

FIGURE 2.—Fairly uniform mononuclear cells comprising sarcoma. Cells appear generally rounded, though cytoplasm is poorly defined. Nuclei display margination of chromatin and usually single nucleolus. Large, clear macrophages present, containing degenerating cells and cellular debris (*arrows*). × 520

FIGURE 3.—Tumor-bearing hamster. Tumor at site of subcutaneous injection is doughnut-shaped, with central ulceration and scabbing. *Note* right axillary lymph-node enlargement. Darkly pigmented area in *left* midback is normal sex spot of male hamster.

FIGURE 4.—Two large tumor cells of peripheral blood. Cytoplasm scanty and difficult to discern. *Left portion* of field shows normal lymphocyte and polymorphonuclear leukocyte. × 1,340





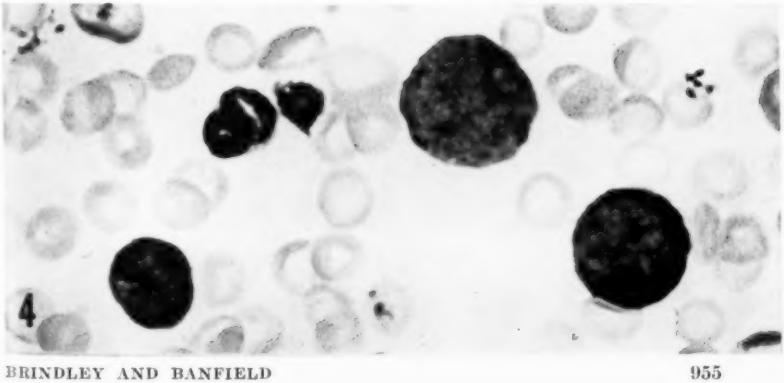
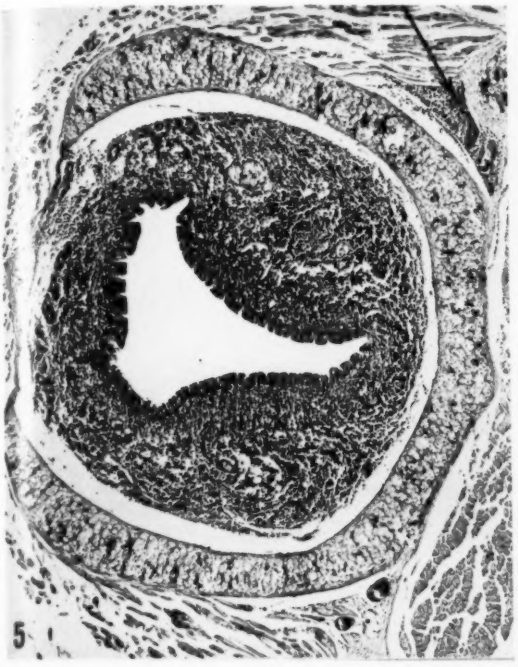


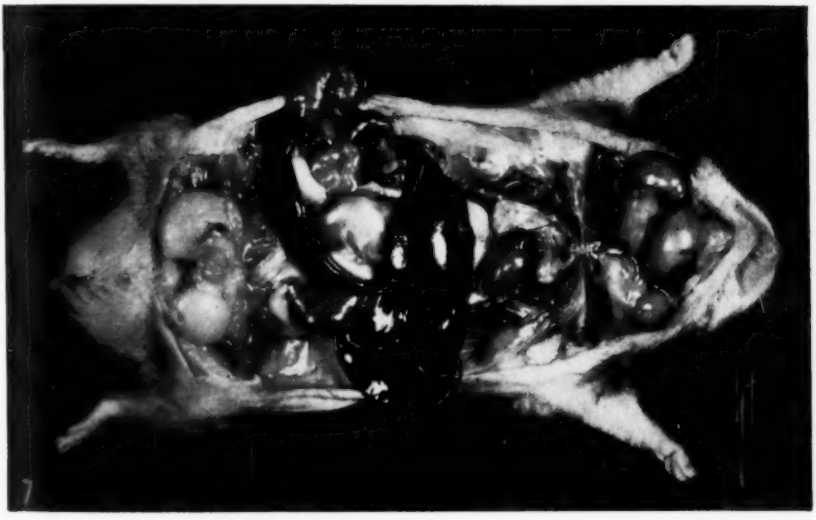
PLATE 126

FIGURE 5.—Larynx of exposed, uninoculated hamster. Mucosa is greatly thickened by tumor. There is one area of perivascular infiltration (*arrow*). × 37

FIGURE 6.—Higher magnification of figure 5. *Note* clear macrophages and one cell in metaphase (*arrow*). × 610

FIGURE 7.—Hamster whose tumor was result of tumor feeding. Cervical and mediastinal lymph nodes, salivary glands, liver, spleen, and kidneys are involved.





Liver Enzymes During Hepatic Carcinogenesis [1]

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SUMMARY

In rats fed a diet supplemented with *N*-2-fluorenylacetamide or *N,N*-dimethyl-*p*-phenylazoaniline, the activity of a number of microsomal and soluble liver enzymes was observed over a 24-week period. During this time the activity of the following enzymatic reactions was estimated periodically: glucuronyl transferase, the *N*-demethylation of morphine, the hydroxylation of acetanilide, tryptophan peroxidase, glutathione reductase, uridinediphosphateglucose dehydrogenase, and the reduction of the 4,5 double bond of ring A of cortisone. The activity of the enzymes, which *N*-demethylate morphine and hydroxylate acetanilide, decreased progressively toward the time when hepatoma formation occurs. In contrast, the glucuronyl transferase and the glutathione reductase showed significant increases in activity. *N*-2-fluorenylacetamide also induced a marked rise in the activity of the uridinediphosphateglucose dehydrogenase.—J. Nat. Cancer Inst. 26: 959–968, 1961.

THE INDUCTION of neoplasia by feeding or topical application of a variety of carcinogens has been studied by many investigators, and the literature contains numerous well-documented observations regarding dietary requirements, cocarcinogens, animals, or strain differences in carcinogenesis. Very little light, however, has been shed on the mechanism of action of chemical carcinogens. A number of these agents, such as the azo dyes, fluorenamines, polycyclic hydrocarbons, and alkylating agents, have been studied quite thoroughly and excellent reviews on the chemistry of these compounds and their effects in relation to the carcinogenic process are available (*1*, *2*).

Even though the actual mechanism of action of these agents remains obscure, many investigators have been attracted by the "enzyme deletion hypothesis" which has been expounded and reviewed in an elegant manner by Potter (*3*). Aside from the phenomenon that transformation of normal into neoplastic cells seems in most instances to be associated

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with a decrease in respiration and an increase in anaerobic glycolysis, little direct evidence has been available to substantiate the enzyme deletion hypothesis or to relate carcinogenesis to it. Comparative enzymatic studies have been concerned with quantitative or qualitative differences between normal and neoplastic cells. Other observations have dealt with enzymatic changes which take place during the induction process. The sum of these investigations failed to yield a clue to a biochemical lesion common to all neoplastic cells (*4*).

The present investigation was stimulated by observations that certain carcinogenic hydrocarbons had a profound effect on the activity of a number of microsomal enzymes (*5–7*). It has been reported that 3,4-benzpyrene (benzo[*a*]pyrene), 3-methylcholanthrene, or 1,2,5,6-dibenzanthracene (dibenz[*a*,*h*]anthracene) administered intraperitoneally induced significant increases in the activity of hepatic microsomal enzymes over a 24- to 48-hour period. The enzyme systems primarily involved are those which catalyze the detoxication of foreign compounds or metabolic alteration of related endogenous products (*8*).

It seemed possible that if a marked stimulation of some of these microsomal enzymes was maintained for a sufficient period, exhaustion of these enzyme systems, or of the systems which synthesize these enzymes, would occur. In that way a cell or its progeny would eventually be deprived of enzymes essential for the elimination of foreign compounds or metabolic waste products. Such a deprivation conceivably could play a significant role in the carcinogenic process. In this study, a number of microsomal enzymes were observed in rats over a period during which hepatomas were induced by two well-established liver carcinogens. To follow a general metabolic pattern of liver enzymes, the levels of a number of nonmicrosomal enzymes were also observed.

METHODS

Groups of 25 Sprague-Dawley male rats weighing from 150 to 180 g were given a special diet (table 1) which was supplemented as follows: Group A diet contained 250 mg/kg of *N*-2-fluorenylacetamide (2-acetylaminofluorene, 2-AAF) in corn oil; Group B diet contained 600 mg/kg of *N*,*N*-dimethyl-*p*-phenylazoaniline (4-dimethylaminoazobenzene, DAB) in corn oil; Group C rats serving as controls received a diet supplemented with corn oil alone. A fourth group comprised of normal animals was maintained on a standard Purina chow diet.

Two animals from each group were killed at specified intervals by decapitation, the livers inspected for gross pathology and weighed. Liver homogenates were prepared in cold isotonic KCl and contained 250 mg of wet liver/ml of homogenate. The homogenate was centrifuged at 9000 $\times$ *g* for 10 minutes, and the supernatant containing the microsomes and the soluble proteins was used for the assay of the *N*-demethylation of morphine, hydroxylation of acetanilide, reduction of the Δ-4 double bond

TABLE 1.—Composition of basic diet*

Ingredient		Percent
Protein	(vitamin-free casein)	12. 0
Starch	(cerelose)	78. 0
Salt mixture	(M-D 185)	4. 0
Vitamin mixture	(mg/kg)	1. 0
B_1	200	
B_2	200	
B_6	1250	
Niacin	200	
Calcium panthothenate	1000	
Cod-liver oil		1. 0
Alpha cell		4. 0
Corn oil		5. 0

*Nutritional Biochemical Corp., Cleveland, Ohio.

in cortisone and of tryptophan peroxidase. For the assay of the glucuronyl transferase systems, the 9000 × *g* supernatant was centrifuged at 78,000 × *g* in a #30 rotor of a Spinco model L centrifuge for 45 minutes. The sedimented microsomes were suspended in isotonic KCl. The soluble supernatant was dialyzed against M/100 phosphate buffer at *p*H 7.0 and served for the assay of uridinediphosphateglucose dehydrogenase (UDPG dehydrogenase), glutathione reductase (GSSG reductase), and glucose-6-phosphate dehydrogenase. Microsomal proteins were estimated by the method of Warburg and Christian (*9*) in N/10 NaOH. Protein concentration of the soluble enzymes was measured similarly. Enzyme assays were performed in duplicate or triplicate on each animal separately, and results in the text-figures indicate the range of values obtained.

Enzyme Assays

Microsomal enzymes

(*1*) *Hydroxylation of acetanilide.*—The incubation mixture contained the following: 1.0 ml of enzyme, 1.0 ml of tris(hydroxymethyl)aminomethane (tris) buffer M/10 *p*H 8.2, 0.4 mg of triphosphopyridine nucleotide, 0.2 ml of 0.5 M nicotinamide, and 0.2 ml of acetanilide (25 μmoles/ml). In each assay, 3 samples containing 0.5 or 1.0 μmole of *N*-acetyl-*p*-aminophenol were carried through the procedure as internal standards. After incubation for 60 minutes at 37° C in an atmosphere of 95 percent O_2 and 5 percent CO_2, 0.5 ml of 60 percent perchloric acid was added to the incubation mixture. After centrifugation, 2.0 ml of the supernatant was heated at 100° C for 20 minutes and the hydrolysate was placed in an ice bath. Three ml of N KOH and 0.5 ml of 2M $KHCO_3$ were then added, and the mixture was centrifuged in the cold. One ml of N/10 HCl was added to 2.0 ml of this supernatant, followed by 1.0 ml of 1 percent phenol (*10*) and 1.0 ml of sodium hypobromite. The samples were allowed to stand for 20 minutes, and the color developed by *p*-aminophenol was estimated spectrophotometrically at 620 mμ.

(*2*) *Glucuronide conjugation.*—Uridinediphosphate glucuronic acid (UDPGA) was prepared enzymatically. For the estimation of glucuronyl transferase and UDPG dehydrogenase activities, 8 ml of the 9000 $\times$ *g* supernatant was removed, diluted to 30 ml, and centrifuged at 78,000 $\times$ *g* for 45 minutes to obtain the differential fractions as previously described by Strominger *et al.* (*11*). Glucuronyl transferase activity was measured by the formation of *o*-aminophenolglucuronide by the method of Levvy and Storey (*12*), as modified by Inscoe and Axelrod (*13*).

(*3*) *N-demethylation.*—The *N*-dealkylation of morphine was determined by the measurement of the formaldehyde formed, according to a modification of the method of Nash (*14*) as described by Cochin and Axelrod (*15*).

Soluble enzymes

(*4*) *UDPG dehydrogenase.*—This was determined according to Strominger *et al.* (*11*).

(*5*) *Glutathione reductase.*—The GSSG reductase activity was estimated by the method of Racker (*16*). The 78,000 $\times$ *g* supernatant was dialyzed against 0.01 M phosphate buffer at *p*H 7.0 for 18 hours at 4° C, and the activity of GSSG reductase assayed on a tenfold diluted solution. The levels of activity were obtained as μmoles of reduced triphosphopyridine nucleotide (TPNH) oxidized/minute/mg protein.

(*6*) *Cortisone reductase.*—Reduction of the 4,5 double bond of ring A of Δ^4-3-ketosteroids was estimated, according to the method of McGuire and Tomkins (*17*), in a mixture which contained 1.0 ml of enzyme, 1.0 ml of *p*H 7.5 tris buffer, 0.1 ml of M/10 $MgCl_2$, and 2.0 mg of reduced triphosphopyridine nucleotide, with 0.05 mg of cortisone (17α, 21-dihydroxy-4-pregnene-3,11,20-trione) as substrate. Activity was estimated by the reduction of the optical density at 240 mμ of methylene chloride extracts of the incubation mixtures after incubation at 37° C for 0, 5, and 15 minutes.

Soluble and microsomal

(*7*) *Tryptophan peroxidase.*—Activity of this enzyme was measured according to the method of Knox (*18*) at *p*H 7.0 in an incubation mixture containing 1.0 ml of the 9000 $\times$ *g* supernatant and 10 μmoles of L-tryptophan as substrate.

RESULTS

After the initiation of the diet feeding, animals in each experimental group showed a slight decrease in body weight (table 2). Throughout the feeding period the weights remained fairly constant, but at the end of the 24th week the control group had gained an average of 36 g, the 2-AAF-fed rats an average of 20 g, and the DAB group 16 g. The liver weights of the control animals remained relatively constant for 24 weeks while liver weights of the 2-AAF-fed animals increased appreciably. A slight increase

in the liver weights of the DAB group was apparent toward the end of the observation period. After 1 week, total microsomal protein/g of wet-liver weight was significantly elevated in the group fed 2-AAF (table 2). This group subsequently showed a progressive decline in the amount of microsomal protein per g of liver until the end of the induction period. After the 14th week, the livers of rats fed 2-AAF had a mottled yellow-gray appearance, with a granular surface. At 24 weeks one rat developed a tumor, 0.5 × 0.5 cm, apparently involving the external ear canal. The livers of rats fed DAB showed a progressively yellow discoloration and, toward the end of the feeding period, some coarse granulation of the hepatic tissue was noted.

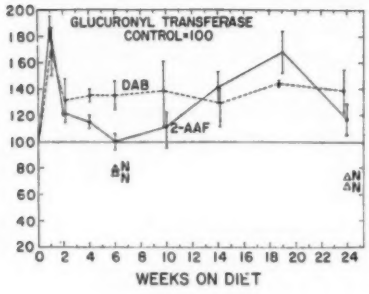
Table 2.—Average body and liver weights and microsomal proteins in control and experimental animals

Time (weeks)	Body weight of animals (g)		
	Controls	2-AAF	DAB
1	201	192	183
2	198	185	176
4	185	173	196
6	210	190	206
10	255	192	192
14	252	175	179
19	255	183	196
24	237	212	199
	Liver weights (g)		
1	8.4	9.5	8.3
2	8.1	7.9	8.9
4	6.8	7.1	8.0
6	7.8	8.6	8.5
10	9.0	8.4	7.9
14	9.5	10.8	8.1
19	10.0	13.1	9.8
24	7.8	14.3	10.9
	Microsomal protein (mg/g wet-liver weight)		
1	48	64	50
2	49	54	48
4	50	40	52
6	50	41	50
10	52	49	50
14	40	34	35
19	54	41	41
24	48	40	45

Glucuronyl Transferase

The conjugation of *o*-aminophenol in animals on the control diet was slightly elevated over those of animals of the same age on the Purina chow diet. The effects of the diets supplemented with azo dye or with 2-AAF showed similar variations from the controls. Within 1 week after initiation of feeding, a marked rise of the glucuronyl transferase activities was observed which fell sharply at the end of the 2d week. Throughout the 2d to 14th weeks the enzymatic activity in each group remained higher than in the controls. A second maximum of activity

was observed in the 19th week and this was followed by a decline in activity. Generally, the changes in enzyme activity induced by 2-AAF were more pronounced than those produced by azo dye (text-fig. 1).



TEXT-FIGURE 1.—Glucuronide conjugation of *o*-aminophenol. The amounts of *o*-aminophenol glucuronide formed by animals on Purina chow diet (ΔN) varied from 0.12 to 0.13 μmole/g wet-liver weight/0.5 hours.

N-Demethylation

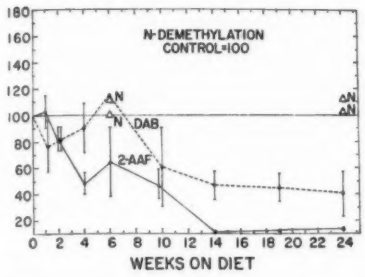
With 2-AAF, a marked and progressive decrease in the liver's ability to dealkylate morphine was observed. At the end of the 14-week period, the enzyme activity had diminished to about 10 percent of the control livers and remained at this level throughout the observation period. DAB induced an initial decrease of *N*-dealkylation activity, but a slight increase above the control levels was noted at the 6th week. Thereafter, the activity decreased progressively to about 40 to 50 percent of the controls. The animals on the control diet showed a decrease of about 10 percent over the whole period when compared to rats fed chow diet (text-fig. 2).

Hydroxylation

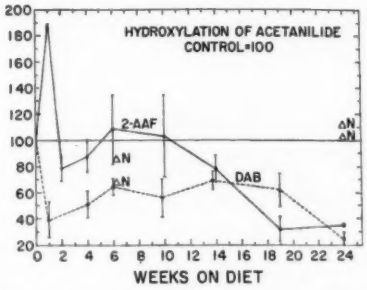
After 1 week of feeding, 2-AAF produced nearly a twofold increase in the hydroxylating activity of the liver which was followed by a decrease in enzyme activity below control levels. From the 6- to 10-week period a recovery of activity to control levels was observed. Toward the end of the experiment a gradual decline of enzyme activity to about 35 percent of control values was apparent. In contrast, azo-dye feeding resulted in a significant initial decrease of enzyme activity and, even though this was accompanied by a moderate recovery of activity, toward the end of the experiment hydroxylation activity had decreased to about 25 percent of the control levels. There were no marked changes in animals on the control and normal diets (text-fig. 3).

Tryptophan Peroxidase

During the first 2 weeks of feeding either carcinogen, a moderate elevation of enzyme activity was observed. For the remainder of the



TEXT-FIGURE 2.—*N*-demethylation of morphine. The amounts of formaldehyde formed by animals on Purina chow diet (ΔN) varied from 5.6 to 6.8 μmoles/g wet-liver weight/1.5 hours.



TEXT-FIGURE 3.—Hydroxylation of acetanilide: The amounts of *N*-acetyl-*p*-aminophenol formed by animals on Purina chow diet (ΔN) varied from 1.53 to 1.64 μmoles/g wet-liver weight/hour.

experiment, small deviations from the control levels were noted, and toward the end of the induction period the activities of both experimental groups were slightly or moderately suppressed (text-fig. 4).

Cortisone Reductase

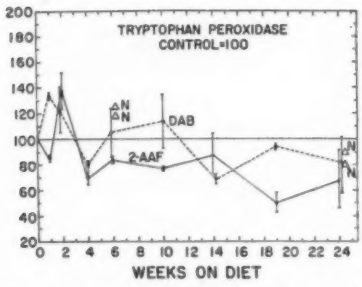
During the first 2 weeks, a moderate elevation in enzymatic activity was observed with either carcinogen. For the remainder of the experiment, a slightly increased level of activity was maintained in the animals fed 2-AAF, while at the end of the 24-week period the azo-dye-fed rats showed values comparable to those of the control animals (text-fig. 5).

Glutathione Reductase

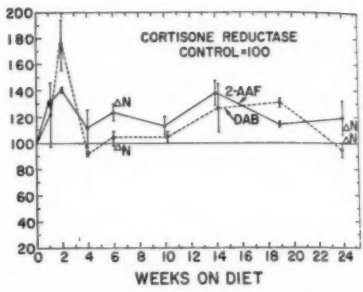
With either carcinogen a slow and progressive increase in GSSG reductase levels seemed to be produced which reached a maximum of more than 200 percent with 2-AAF and 50 percent with the azo dye, near the 14-week period. Thereafter activity approached that of the control animals until the end of the experiment (text-fig. 6).

UDPG Dehydrogenase

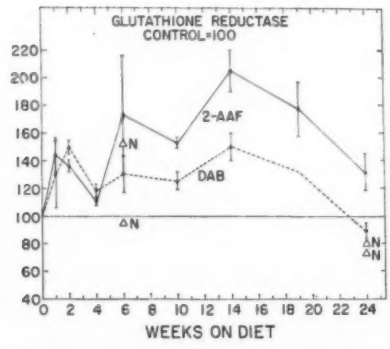
With DAB, only moderate changes in the activity of this enzyme were observed. With 2-AAF, however, a marked rise in enzyme activity was noted at the 14th and 19th weeks. Toward the end of the experiment, the stimulation produced by 2-AAF seemed to subside, even though activity was still about 40 percent higher than in the control animals, which showed a 12 to 16 percent elevation of activity when compared to normal animals on the regular diet (text-fig. 7).



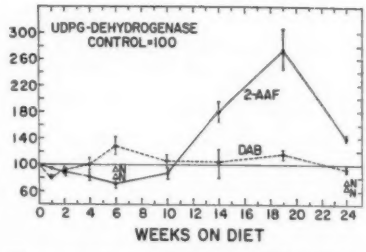
TEXT-FIGURE 4.—Tryptophan peroxidase. The amounts of kynurenine formed by animals on Purina chow diet (ΔN) varied from 19.8 to 22.9 μmoles/g wet-liver weight/hour.



TEXT-FIGURE 5.—Cortisone reductase. The amount of cortisone reduced at the 4,5 double bond of ring A by animals on Purina chow diet (ΔN) varied from 0.26 to 0.37 μmole/g wet-liver weight/0.25 hours.



TEXT-FIGURE 6.—Glutathione reductase. The amount of reduced triphosphopyridine nucleotide (TPNH) oxidized by animals on Purina chow diet (ΔN) varied from 0.034 to 0.053 μmole/mg protein/minute.



TEXT-FIGURE 7.—Uridinediphosphate-glucose dehydrogenase. The activity of this enzyme in animals fed Purina chow diet varied from 20 to 27 units per minute (*11*).

DISCUSSION

The gross changes in body weights, liver weights, and changes in the appearance of the livers were considered consistent with previously published data on animals fed diets containing fluorenamines or carcinogenic azo dyes. The marked increase in enzyme activity obtained with fluorenylacetamide for the glucuronyl transferase and for the hydroxylation of acetanilide after 1 week on the diet could represent an "induction" phenomenon similar to the rapid increases obtained after intraperitoneal injection of certain hydrocarbons (*7*). It was previously observed that these two enzymes will respond to an intraperitoneal injection of 2-AAF

with a slight increase in activity (*19*). The consistently elevated glucuronyl transferase activity of the animals fed the carcinogens might indicate that the effects on microsomal enzymes induced by hepatocarcinogens might be quite selective, especially in view of the fact that both the hydroxylating system and the *N*-dealkylation system were markedly depressed from the 14th week on. Whether or not the elevated glucuronyl transferase activity can be considered as a compensatory mechanism in the over-all detoxication functions, however, remains questionable. The marked loss in the ability of the microsomal enzyme systems to *N*-demethylate morphine and to hydroxylate acetanilide was considered significant. Studies in other laboratories had shown that the *N*-demethylating (*20*) or hydroxylating (*21*) activity of induced hepatomas is either absent or very low. There is no evidence now to implicate an alteration of any microsomal enzyme system in the carcinogenic process, but the activities of both enzymes decreased progressively toward the time when hepatoma formation occurred.

Aside from some initial stimulation, the activities of tryptophan peroxidase and of cortisone reductase showed lesser deviations from the control levels. In contrast, the glutathione reductase levels showed a progressive rise from the 1st month to the 14th week with either carcinogen. A similar, but more pronounced, stimulation was observed in the activity of the UDPG dehydrogenase levels in livers from 2-AAF-fed rats. The significance of this response is not evident, though it could be argued that in the UDPG dehydrogenase the stimulation is a sequel to, or initiated by, an increased utilization of UDPGA by the glucuronyl transferase system. Considering all systems studied here, the over-all impression was that the systems which require molecular oxygen showed a tendency toward a progressive decline in activity. Since the most strongly inhibited enzyme, the *N*-demethylating system, also depends on TPNH, occasional assays for glucose-6-P-dehydrogenase were carried out. No decrease was detected in the activity of G-6-P-dehydrogenase toward glucose-6-phosphate, and it was assumed that in the presence of TPN a sufficient amount of reduced pyridine nucleotide was available for the *N*-demethylation of morphine.

The effects of 2-AAF and DAB on the hydroxylating and *N*-demethylating activity of the liver could be conceived of as an enzyme deletion. If the significance of either system in the maintenance of hepatocellular function were established, the data presented here might be considered to support the enzyme deletion hypothesis. That all the other enzymes investigated showed essentially normal, or even elevated, levels might suggest the hypothesis that the carcinogens induce neoplasia by interference with rather specific mechanisms which are required for the resynthesis of certain enzymes essential for the maintenance of a normal cell.

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Influence of the A^y Gene on Mammary-Gland Tumors, Hepatomas, and Normal Growth in Mice [1]

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SUMMARY

Hybrid (C3H × Y)F_1 mice that were genetically uniform except for segregation at the agouti locus (1/2 A^yA, yellow, and 1/2 Aa, agouti) were studied in respect to body size, occurrence of mammary tumors in the females, and occurrence of hepatomas in the males. An effect of A^y increasing susceptibility to mammary tumors was noted in the age at which the tumors arose in the virgin females. The average tumor age of the Aa virgins was 15 months while that of the A^yA virgins was only 8 months. Breeding eliminated this difference, for the average tumor age in both genotypic groups of breeders was 8 months. The final incidence in all 4 groups was or approximated 100 percent. In addition to adenocarcinomas of type A or type B there was a distinct group of a type rarely seen in other strains or hybrids that has been designated as adenocarcinoma, type Y. All but one of those of this type occurred in breeders. A^y increased susceptibility to spontaneous hepatomas in the males. The incidence in both genotypic groups was high, but it was higher in the A^yA males than in the Aa males, and the A^yA males had a significantly higher average number of hepatomas than the Aa males. Males treated with CCl_4 failed to show any carcinogenic effect of this compound in increasing occurrence of hepatomas in these susceptible hybrids, and the A^y gene did not significantly increase the occurrence of hepatomas in these treated males. A^y increased body weight by approximately 10 g in the males and approximately 15 g in the females; it significantly increased total-body length in both males and females, and it significantly increased weight of gastrocnemius muscle in the females. In the combined genotypic groups of males, a significant positive correlation was found between number of spontaneous hepatomas and body weight, total-body length, and length of femur. Relationships between normal growth and neoplasia are discussed.—J. Nat. Cancer Inst. 26: 969–983, 1961.

ONE OF the most interesting genes of the mouse is lethal yellow (A^y) of the agouti series in linkage group V (*1*, *2*). When homozygous, it has a lethal effect on the early embryo (*3–5*), but heterozygous animals display a yellow coat in the presence of the color gene, and also have an increase in body weight with obesity and in body length and length of tail as compared with sibs without the gene (*6*, *7*). This increase in body weight is

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associated with an increased food intake with reduced energy requirements (*8*).

In addition, A^y has been shown to increase the occurrence of pulmonary tumors. The effect has been observed when the tumors arose spontaneously and when they were induced with methylcholanthrene, urethan, or nitrogen mustard, though no effect was noted when the tumors were induced with dibenz[*a*,*h*]anthracene (*9*, *10*). There was evidence that the increase in pulmonary tumors by the gene might in some way be related to its effect on normal growth.

In 1934, Little (*11*) reported an effect of A^y on occurrence of mammary tumors in hybrid females from crosses between high-tumor strain DBA females and strain Y males. Mammary tumors appeared at an earlier age in the yellow females than in the nonyellow females, but, contrary to what would have been expected, the final incidence in the nonyellow females was higher than that in the yellow females. It was thought that these results were probably owing to the fact that the yellows passed through their reproductive cycle earlier than did the non-yellows.

It seemed that it would be worthwhile to extend the study of the effect of A^y on tumor formation with hybrids between strain C3H females and yellow strain YBR males. In addition to providing a different system in which to study the effect of the gene on mammary tumors, the hybrid males, being highly susceptible to hepatomas, would provide a test for the effect of A^y on hepatomas. Further studies on the effect of A^y on normal growth and the correlation of the effect of A^y on normal growth with its effect on the occurrence of the neoplasms were also desirable.

MATERIALS AND METHODS

The study was carried out on (C3H $\times$ Y)F_1 mice produced by mating strain C3H females with yellow strain YBR males. The C3H females were from our colony of strain C3H mice that we have used extensively in studies on mammary tumors and hepatomas. The incidence of mammary tumors in both breeding and virgin females of this strain approaches 100 percent with an average tumor age of a little less than 8 months in the breeders and between 10 and 11 months in the virgins (*12*). Recently we have also reported a very high incidence of spontaneous hepatomas in the males of the strain, which ranged from 72 to 85 percent for the various sublines, when fed NCI pellets (*13*). The YBR males were from our colony of strain YBR that we have used in previous studies of linkage between susceptibility to tumors and the lethal yellow gene. The recorded incidences of mammary tumors and of hepatomas are low in this strain, but since the animals die at a relatively early age with amyloidosis and the accompanying nephritis, an accurate measure of their susceptibility to tumor formations has not been possible.

These (C3H $\times$ Y)F_1 hybrids offered an ideal system for studying the effect of a specific gene on the occurrence of tumors. Although they would be expected to be heterozygous at many loci, all would be genetically alike except for the difference at the agouti locus and possibly also at closely linked loci. The C3H females being highly inbred would theoretically be homozygous at all loci, and the YBR males also being highly inbred (the males used were of the F_{63-67} brother and sister inbred generations) would theoretically be homozygous at all loci except at the agouti locus where there would be forced heterozygosis because of the lethal effect of A^yA^y. All yellow mice of strain YBR are A^ya. Since the C3H females were AA, one could expect half the F_1 hybrids to be yellow A^yA and half agouti Aa. In addition to this difference there would, of course, be differences associated with the sex chromosomes, but these differences did not interfere with the present analyses of the effect of A^y, since the effect on mammary tumors was limited to females and that on hepatomas was observed in males.

Throughout the study all mice were fed NCI pellets, the formula of which has been published (*13*), and were given tap water *ad libitum*. They were housed in an air-conditioned room maintained at approximately 78° F.

All the F_1 hybrids were weaned when they were 4 weeks of age, individually identified, and classified as to whether they were yellow, A^yA, or agouti, Aa. They were separated as to sex and housed in plastic cages with 8 mice to the cage, except for approximately half the females which were studied as breeders and the Aa males to which they were bred. These were mated in cages each containing 5 females and 1 male, the females being isolated when pregnant and returned to the males when the litters were weaned.

The breeding and virgin females were observed regularly for the occurrence of mammary tumors. Soon after the tumors appeared or when the females became moribund from other causes they were killed and examined. The occurrence of all tumors and other lesions was recorded, and all tumors were fixed in Fekete's modification of Tellyesniczky's fixative (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), sectioned, and stained with hematoxylin and eosin for histologic classification.

The males were divided into two groups, one for ascertaining whether or not A^y had any influence on hepatomas induced with carbon tetrachloride and the other for the observation of any effect of this gene on spontaneous hepatomas. The treatment with CCl_4 was similar to that used by Edwards (*14*) who demonstrated that this compound was effective in inducing hepatomas in strains C3H and A mice. The mice were fed 0.1 cc of 40 percent CCl_4 in olive oil by stomach tube twice a week for 16 weeks, beginning at 2 months of age. At 14 months of age all remaining males of the treated group were killed and examined for hepatomas and other tumors, and at 16 months all remaining males of the spontaneous tumor group were killed and examined. Any males of either

group that became moribund or were found dead before these ages were likewise examined for tumors. Gross observations of the hepatomas and other tumors were recorded, and all questionable tumors were fixed, sectioned, and stained as were the mammary tumors for histologic classification.

Sample groups of 19 A^yA virgin females, 29 *Aa* virgin females, 14 A^yA nontreated males, and 14 *Aa* nontreated males were weighed at weaning and at subsequent weekly intervals for the construction of growth curves. All other males were weighed at 10 months of age and again at the time of autopsy. Also at autopsy total-body length (from the tip of the nose to the tip of the tail), length of the left femur, and weight of the left gastrocnemius muscle that had been dissected out were recorded for all males. In previous work these measurements have served as adequate measures of skeletal and muscular development (*15*). (On analysis of the data, however, it appeared that the muscle weights of these males had been greatly influenced by old-age factors and they were therefore omitted.) To observe correlations between genotype and these body measurements before the measurements could be influenced by factors associated with old age, sample groups of each genotype and each sex were killed at 4 months of age and these body measurements for each animal were recorded.

RESULTS

Mammary-Gland Tumors

The occurrence of mammary-gland tumors in the breeding and virgin females of both genotypes is recorded in table 1. These (C3H $\times$ Y)F_1 hybrids were very susceptible to mammary tumors, and, since all of them had obtained the mammary-tumor agent from their C3H mothers, a very high tumor incidence of 96 percent and above was observed in all 4 groups. Thus, no effect of A^y on the incidence of mammary tumors could be detected. Furthermore, no effect of the gene on the tumor age of the breeding females was observed. The average tumor age in both the A^yA and *Aa* breeding females was relatively low. A highly significant effect of the gene on tumor age was noted in the virgin females. The average tumor age for the A^yA virgin females was comparable with that of both groups of breeders, but the average tumor age of the *Aa* virgin females was approximately twice that of the A^yA virgins.

These data are in agreement with those published earlier by Little (*11*) in that they show that A^y caused the mammary tumors to occur at an earlier age, though in these hybrids the difference was limited to the virgin females. The data do not confirm his observations that the yellow females in which the tumors appeared earlier had a lower final incidence than the nonyellow females. In his study, however, the yellow females that did not develop tumors died at an earlier age than did the nonyellow females dying without tumors.

TABLE 1.—Occurrence of mammary-gland tumors in (C3H × Y)F_1 females

Genotype	Females	Number of females	Incidence of mammary tumors (percent)	Average tumor age (months)	Average age females died without tumors (months)
A^yA	Breeders	106	98	7.9	9.5
Aa	Breeders	107	97	8.4	7.0
A^yA	Virgins	88	100	8.1	—
Aa	Virgins	112	96	15.4	17.3

The mammary-gland tumors observed in these F_1 females are listed as to histologic type in table 2. The total number of tumors exceeded the number of females with tumors because some females had multiple tumors. The majority of the tumors were adenocarcinomas of either Dunn's type A with definite acinar structure or her type B with the cells lying in sheets or in cords between spaces (*16*). Two carcinosarcomas were found in A^yA virgin females. Three tumors were listed as "miscellaneous," a term used to indicate tumors that cannot be fitted into a previously described morphologic group. These 3 tumors were similar. All were composed of strands of cuboidal epithelial cells resting on a delicate connective-tissue membrane. The epithelial strands surrounded irregular spaces containing a sparse amount of a homogeneous, faintly eosinophilic material. Many of the epithelial cells were vacuolated, and material within the vacuoles was similar to the material within the spaces. The over-all structure presented a lacelike appearance under low magnification (fig. 1).

TABLE 2.—Mammary tumors of (C3H × Y)F_1 females classified according to histologic type*

Group	Number of tumors	Type A (No.)	Type B (No.)	Type Y (No.)	Carcinosarcoma (No.)	Miscellaneous (No.)
A^yA (breeders)	178	75(4)	86(5)	16(1)	—	1
Aa (breeders)	170	53(3)	105(6)	10	—	2
A^yA (virgins)	165	55	108(7)	—	2	—
Aa (virgins)	146	81(2)	64	1	—	—

*Numbers in parentheses indicate number with secretory activity.

On studying this lot of tumors we soon began to realize that the mammary tumors of these (C3H × Y)F_1 hybrids contained a group of tumors of a type rarely seen before (fig. 2). These tumors were composed of cuboidal epithelial cells lining irregular angular spaces and clefts. The epithelial strands were separated by connective tissue containing abundant collagen staining pink with the van Gieson stain. A photograph of a comparable tumor has been published by Dunn (*16*) who listed it as a rare tumor of an unclassified type but did not indicate in what strain or strains it had been observed. In the present lot there were 27 of these tumors that seemed to form a definite class. Since we could not recall

having observed them in strain C3H females but only in hybrids resulting from outcrossing to strain YBR, we have classified them as adenocarcinoma, type Y. It would be interesting to induce mammary tumors in YBR females to see if many of them fall in this class.

One of the remarkable things about this group of tumors that we are classifying as type Y is that all but one occurred in the breeding females. This suggests a hormonal influence in their production. The average age at which the tumors of this type occurred was not significantly different from that of the whole lot, and there was no evidence of any influence of the A^y gene on their occurrence.

The occurrence of this special type of mammary tumors in these particular hybrids emphasizes that, while the tumors of no specific strain or hybrid are limited to a particular class, often particular types occur in specific strains or hybrids with a greater frequency than in other groups. For example, we have observed the usually rare type C tumors in relatively high frequency among tumors of (C57BL $\times$ C3Hf)F_1 females and their reciprocals (*12*), and Andervont and Dunn (*17*) observed quite a number of them in (BALB/c $\times$ C3H)F_1 females. Adenoacanthomas occur in a relatively high frequency in strain C3Hf females without the mammary-tumor agent. Thus, while no type of mammary tumor appears to be limited to a specific strain or hybrid, some identification of strain or hybrid can be made through a listing of all types found and the proportions of each type.

In the (C3H $\times$ Y)F_1 females certain tumors other than the mammary tumors were also observed. Of the 106 A^yA breeding females, 1 had a hepatoma and 1 had a pulmonary tumor. Of the 97 *Aa* breeding females, 1 had a papilloma of the skin. Of the 88 A^yA virgins, 12 had hepatomas at an average of 8.5 months, and 1 had a lymphoma of the thymus. Of the 113 *Aa* virgins, 15 had hepatomas at an average age of 18.3 months, 2 had hemangioendotheliomas of the liver, 1 had a sarcoma of the cervix, 2 had reticulum-cell neoplasms, and 1 had a lymphoma of the thymus.

Hepatomas

Comparison of the genotypic groups in respect to the occurrence of hepatomas in the males can be made from the data in table 3. Data for the spontaneous hepatomas were particularly significant. We had previously observed a high incidence of hepatomas in our C3H males (*13*) and the incidence in these (C3H $\times$ Y)F_1 males was even higher. Of the 63 A^yA nontreated males autopsied at 16 months of age, all had hepatomas, and of the 94 *Aa* nontreated males also autopsied at 16 months, 88.3 percent had hepatomas. Comparison of these incidences, though both are high, indicates that A^y increases susceptibility to hepatomas just as it increases susceptibility to mammary and pulmonary tumors. This difference is confirmed by the difference in average number of hepatomas in each group. The A^yA males had an average of 3 hepatomas, whereas the *Aa* males had an average of only 2. The difference between these means is significant at the 1 percent level.

TABLE 3.—(C3H × Y)F_1 males classified according to genotype with occurrence of hepatomas and averages for certain body measurements

Genotype	Number of males	Age (months)	Treatment	Incidence of hepatomas (%)	Average No. hepatomas	Average body weight (g)	Average body length (cm)	Average length left femur (cm)
A^vA	63	16	None	100	3.0	47.7 (53.5) *	21.9	1.66
					$t = 4.280$ $P<0.01$	$t = 3.143$ $P<0.01$	$t = 10.354$ $P<0.01$	$t = 4.288$ $P< 0.01$
						$t = 14.583$ $P< 0.01$		
Aa	94	16	None	88.3	2.0	45.3 (46.0) *	21.0	1.64
A^vA	53	14	CCl_4	84.9	1.6	47.6	21.3	1.65
					$t = 0.956$ $0.3<P<0.4$	$t = 7.142$ $P< 0.01$	$t = 3.257$ $P<0.01$	$t = 1.129$ $0.2<P<0.3$
Aa	34	14	CCl_4	79.4	1.4	41.4	20.9	1.66

*Weights in parentheses were recorded at 10 months of age.

In the group treated with carbon tetrachloride the incidence of hepatomas in the A^vA was slightly higher than that in the *Aa* males, and the average number of hepatomas in the A^vA males was slightly higher than that in the *Aa* males, but these differences were not statistically significant. These groups, however, had fewer animals than the nontreated groups.

These treated males were autopsied at 14 months of age, and at that age the incidence of hepatomas was less than that in the nontreated group autopsied at 16 months, and the average number of hepatomas was likewise less. Thus the data failed to show any carcinogenic effect of this compound in these highly susceptible hybrid males.

Tumors other than hepatomas in these treated and nontreated males killed at 14 and 16 months, respectively, included pulmonary tumors in 5 A^vA nontreated males, in 9 *Aa* nontreated males, in 3 A^vA treated males, and in 3 *Aa* treated males, and hemangioendotheliomas in the livers of 2 of the *Aa* treated males.

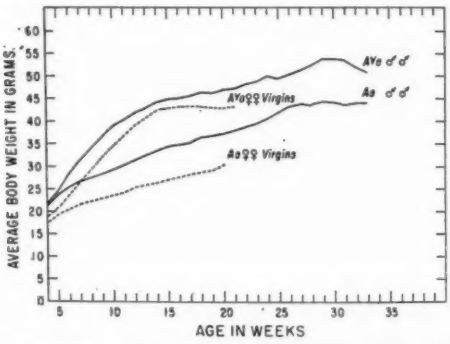
In addition to the nontreated males killed at 16 months of age, 38 A^vA nontreated males were autopsied when found dead or moribund at an earlier age, and 25, or 66 percent, of these had hepatomas. Of the *Aa* nontreated males, 32 were autopsied before 16 months of age, and 19, or 59 percent, had hepatomas. These data are not out of line with those from the nontreated males killed at 16 months in showing an effect of A^v on occurrence of hepatomas, but definite comparison cannot be made, since the ages in the two genotypic groups were variable. Of these 38 A^vA nontreated males autopsied before 16 months, 2 had hemangioendotheliomas in the liver, 2 had reticulum-cell neoplasms, and 3 had pulmonary tumors. Of the 32 *Aa* nontreated males autopsied before 16 months, 2 had hemangioendotheliomas in the liver, 1 had a pulmonary tumor, 1 a thymic lymphoma, and 1 a lymphocytic leukemia.

Of the A^vA males treated with CCl_4, 15 had to be autopsied before the 14 months, many of these at an early age because of the ill effects of the treatment; 2 of these had hepatomas and 1 a pulmonary tumor. Six *Aa* treated males had to be autopsied before the 14 months, but none of these had a tumor.

Body Weights and Measurements

The effect of the A^v gene on increasing body weight can be noted in the growth curves for both males and females shown in text-figure 1. Although the A^vA mice weighed only slightly more than the *Aa* mice at weaning age, the A^vA animals gained weight more rapidly than the *Aa* animals, until the A^vA males weighed approximately 10 g more than the *Aa* males, and there was an even greater difference between the A^vA and the *Aa* females. In old males, however, this difference decreased presumably as the yellow males lost adipose tissue (table 3).

Data from the other body measurements, as well as body weight from the sample groups killed at 4 months of age to avoid old-age factors, are given in table 4. At this age, body weight of the A^vA mice was significantly



TEXT-FIGURE 1.—Growth curves of (C3H × Y)F_1 males and females.

greater than that of *Aa* mice in both sex groups. The body length of A^yA mice was also greater than that of *Aa* mice in both males and females. A significant difference in weight of the left gastrocnemius muscle was noted in the females, the A^yA females showing greater muscular development than the *Aa* females, but the A^yA males were not significantly different from the *Aa* males in muscle weight. There was not a significant difference between the two genotypic groups in respect to length of femur in either sex. Thus, the A^y gene not only increased body weight where an increase in adipose tissue could be a factor, but also increased body size as indicated in total-body length where skeletal development could be a factor and also in muscular development noted in the females.

The effect of the A^y gene on increasing body weight could still be noted at the time the groups of males were autopsied at 14 and 16 months of age (table 3). The differences at these ages were not as great as noted at younger ages, but they were statistically significant. Likewise, a significant increase in body length due to the A^y gene could also be noted in these older males. The A^yA males autopsied at 16 months had significantly longer femurs than the *Aa* males had, but this difference was not confirmed by the group autopsied at 14 months or by the group autopsied at 4 months.

Pearson product-moment coefficients of correlation between the number of hepatomas and the various body measurements were computed for the A^yA and *Aa* males combined in both the treated group killed at 14 months and the nontreated group killed at 16 months (table 5). As can be noted in the table, no significant correlations were obtained in the treated males. Significant positive correlations were found, however, in the nontreated males between the number of hepatomas and body weight, total-body length, and length of femur. The correlation was higher between number of hepatomas and body weight recorded when the animals were 10 months of age than between number of hepatomas and body weight at the time of autopsy when they were 16 months of age. This could be expected, since at 16 months old-age factors could greatly influence the weight of some animals.

TABLE 4.—Average body measurements of (C3H × Y)F_1 mice autopsied at 4 months of age.*

Genotype	Sex	Body weight (g)	Body length (cm)	Weight left gastrocnemius muscle (g)	Length left femur (cm)
A^yA	♂	46.2 (20)	20.5 (20)	0.1980 (20)	1.61 (19)
		$t = 5.455$, $P<0.01$	$t = 2.376$, $0.01<P<0.02$	$t = 1.002$, $0.3<P<0.4$	$t = 0.1083$, $P>0.9$
Aa	♂	39.9 (16)	20.1 (16)	0.2016 (16)	1.62 (16)
A^yA	♀	44.5 (31)	19.8 (31)	0.1652 (31)	1.60 (31)
		$t = 16.360$, $P<0.01$	$t = 7.251$, $P<0.01$	$t = 5.616$, $P<0.01$	$t = 1.847$, $0.05<P<0.1$
Aa	♀	30.8 (23)	19.1 (22)	0.1533 (23)	1.58 (23)

*Figures in parentheses indicate number of mice.

TABLE 5.—Coefficients of correlation between number of hepatomas and the various body measurements in combined A^yA and Aa (C3H × Y)F_1 male mice

Group	Age (months)	Hepatomas—body length	Hepatomas—length of left femur	Hepatomas—body weight	Hepatomas—body weight (10 months)
Nontreated	16	$N = 155$ $r = +0.21$ $t = 2.674$ $P < 0.01$	$N = 154$ $r = +0.19$ $t = 2.383$ $0.01 < P < 0.02$	$N = 157$ $r = +0.17$ $t = 2.106$ $0.025 < P < 0.05$	$N = 157$ $r = +0.42$ $t = 5.798$ $P < 0.001$
Treated with CCl_4	14	$N = 83$ $r = -0.064$ $t = 0.577$ $0.5 < P < 0.6$	$N = 85$ $r = +0.02$ $t = 0.182$ $0.8 < P < 0.9$	$N = 76$ $r = -0.02$ $t = 0.155$ $0.8 < P < 0.9$	—

DISCUSSION

For some time we have been interested in the relationship between normal growth and the occurence of neoplasms and in the effect of specific genes on both. Cancer is a disease of the healthy individual. It has long been observed that to get the maximum tumor response, one keeps his experimental animals as healthy as possible under the conditions of the experiment. As a general rule, factors that increase normal growth, or make a more robust animal, increase the occurrence of neoplasms, and factors that inhibit normal growth decrease the occurrence of neoplasms. Tannenbaum and Silverstone (*18*, *19*) have shown that calorie-restricted diets decrease the occurrence of a number of kinds of neoplasms in mice, and diets high in protein increase the occurrence of tumors. Larsen and Heston (*20*) found a decrease in pulmonary tumors in strain A mice fed a calorie-restricted diet. We found that C3H mice fed NCI pellets had a higher incidence of hepatomas than those fed Purina chow (*13*). Neither diet could be called a deficient diet, and the NCI diet contained nothing that would generally be considered as carcinogenic, but the mice fed the NCI pellets weighed more than those fed the chow. Anderson (*21*) has recently reported an increase in incidence and pathogenesis of ocular squamous carcinoma in cows with an increase in nutritional level. One of the better-defined approaches to the problem in man has been in the constitutional studies carried out by Sheldon and coworkers (*22*). They have found that breast cancer occurs more frequently in women with strong muscular and skeletal development than in thin women or in extremely obese women.

In our studies on pulmonary tumors in the mouse we have been interested in the fact that the A^y gene that increased occurrence of pulmonary tumors increased body weight, whereas 6 other genes that were associated with a decrease in pulmonary tumors were also associated with a decrease in body weight. The irregular gene was the gene for obese (*ob*) that decreased the occurrence of pulmonary tumors while it greatly increased body weight (*15*). However, the obese mice were smaller than their normal sibs in respect to total-body length, length of femur, and weight of gastrocnemius muscle, and in the combined data there were positive correlations between the occurrence of pulmonary tumors and these 3 measurements.

The present study extended this line of observations to mammary tumors and hepatomas in the mouse. This A^y gene that increased body weight, body length, and, in the females, muscle weight, also increased susceptibility to mammary tumors, as indicated by a reduction in tumor age in virgin females, and increased susceptibility to spontaneous hepatomas.

Further studies of correlation between occurrence of mammary tumors and these body measurements could not be made because the females were of varying ages when their tumors appeared and when they were autopsied, and some were in poor condition because of their large tumors. Further-

more, some of the measurements on the old males with hepatomas, particularly muscle weight, were not highly satisfactory because of the poor condition of some of the animals. This is the same problem with which those attempting to carry out constitutional studies on older human beings with neoplasms have been confronted. However, it was found that within the limitations of the data, where significant correlations were found, *i.e.*, between number of spontaneous hepatomas and body weight, body length, and length of femur, the correlations were positive, which is in agreement with other published observations.

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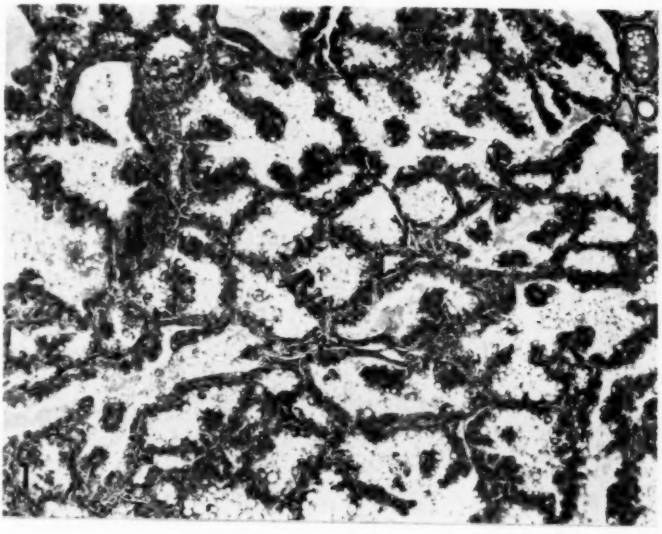
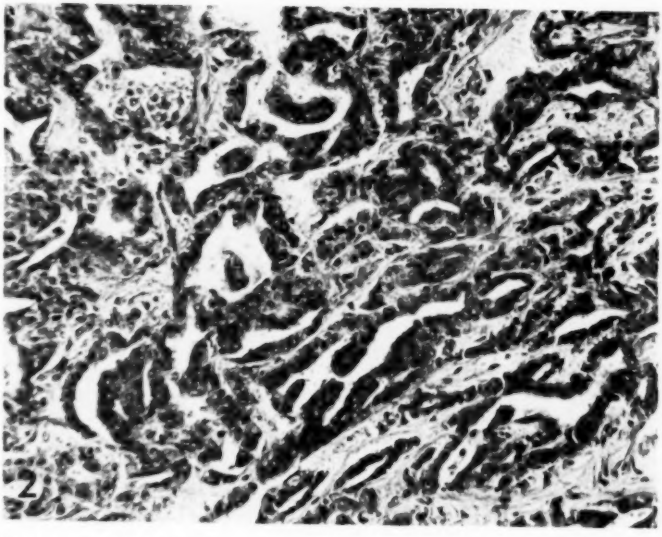
PLATE 127

FIGURE 1.—"Miscellaneous" mammary tumor of unclassified type, from *A*^{v}*A* (C3H × Y)F_1 breeding female 7 months of age. Hematoxylin and eosin. × 110

FIGURE 2.—Mammary tumor with epithelial cells surrounding angular spaces and with large amount of stroma, classified as adenocarcinoma, type Y, from *A*^{v}*A* (C3H × Y)F_1 breeding female 9 months of age. Hematoxylin and eosin. × 210





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Mechanism of Experimental Tumorigenesis. VIII. Ultrastructural Alterations in Mouse Epidermis Caused by Carcinogen Administered Orally [1, 2, 3, 4]

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SUMMARY

An electron microscopic study of the effect of orally administered 7,12-dimethylbenz[*a*]anthracene (DMBA) on the epidermis of the back of the mouse was studied on healthy female mice of a tumor-resistant strain. As in previous experiments with locally applied DMBA, the pattern appeared to agree with that seen in light microscopy of the same material, and contained the histologic criteria generally accepted in classical pathology as characteristic of the effect of a carcinogen upon the mouse epidermis. The electron microscopic features were: (*a*) abnormalities in cell surface-to-cell surface contact, in which combined light, electron, and polarization microscopy indicated disturbances in the detachment of epidermal cells; (*b*) development of new profiles such as a crown of thornlike pattern in the cytoplasmic periphery, which in polarization microscopy appeared to be composed of brilliant anisotropic material; (*c*) disturbances in keratinization and the appearance of immature degradation products and cellular remnants in places where they are not seen normally and in nonspecific states; (*d*) accumulation of globular, electron-dense inclusion bodies intracristally within the internal membranes of the mitochondria even in the basal cells, and (*e*) a characteristic variability in degree and quality of the changes in various places. The character of the alterations was independent of the dose of DMBA and was not affected by the hair follicular cycle. The results of control experiments, as well as light microscopy studies of serial sections from internal organs, rule out the possibility that these changes might depend on irrelevant external carcinogen contamination or on a hypothetical general toxicity of the carcinogen under the present experimental conditions.—J. Nat. Cancer Inst. 26: 985–1009, 1961.

SKIN TUMORS in the mouse back can be provoked by administration of the carcinogen, used as tumor initiator, intravenously, intraperitoneally, or orally, and then by treatment of the skin locally with the tumor promoter (*1–3*). Carcinogens given orally bring about distinct changes in the skin of female mice irrespective of the carcinogen used, the dose

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[4] Paper VII of the same series will appear in *Acta path. et microbiol. scandinav.*

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(the lowest single dose so far tested was 100 γ), the time elapsed, or the phase of the hair follicular cycle at the moment of biopsy. These changes intimately resemble those caused by the same carcinogen applied locally (*4*).

The purpose of the present study is to show that 7,12-dimethylbenz[*a*]-anthracene given orally to mice of a resistant strain causes similar ultrastructural alterations in the epidermis as when applied locally (*5*).

MATERIALS AND METHODS

The general experimental procedures appear in our previous papers, *e.g.*, in this series.

Female mice, 2 to 3 months old, of the randomly bred, tumor-resistant RA strain of this laboratory were used. They received food and water *ad libitum*. In some experiments the animals were housed 5 in each cage, in others they lived alone in glass cages that permitted free movement. Numerous controls were carried out on single animals alone in specially constructed glass tubes; this procedure precluded all movements of the mouse, including licking of the fur, and also ruled out all external sources of contamination.

The carcinogen was 9,10-dimethyl-1,2-benzanthracene[6] (DMBA) from L. Light & Co., Colnbrook, England. The carcinogen was dissolved in 0.25 ml of nonfluorescent light paraffin or in 0.15 ml of undiluted Carbowax 400 from Carbon and Carbide Chemicals Co., New York, and the solution was given by gastric instillation into the stomach of the fasting mouse. DMBA, 100, 200, 400, 600, 800 γ, and 1.6 mg per dose, was given only once, or on 5 successive days. Details of the experiments appear in respective paragraphs that follow.

The hair from the biopsy area on the back of the mice was not cut until a few seconds before they were killed. The biopsies were taken between 8 and 9 AM to avoid the possible influence of diurnal fluctuations of the mitotic activity in the epidermis. The hair follicular cycle (anagen, catagen, or telogen) was observed as previously. Preparations for light microscopic controls were fixed in Susa mixture, embedded in paraffin, and stained with hemalum and eosin. Because the mouse epidermis is extremely sensitive, even to mild irritants such as painting with water, all nonspecific inflammatory changes possibly encountered in light microscopy were carefully analyzed. Some specimens of each series were left unstained for examination in polarized light in order to demonstrate that the localization of the electron-dense profiles of the altered epidermal cells found in electron microscopy corresponded to the anisotropy observed under the polarization microscope (*cf. 6*). In addition, the liver, lungs, stomach, the whole intestine, kidneys, and spleen were fixed in the same solution, embedded in paraffin, cut serially, and examined under the conventional microscope.

[6] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

Because light microscopic examination (*cf. 4*) revealed that the most characteristic changes occurred within the pilosebaceous apparatus and in the interfollicular epidermis (IFE) adjacent to it, the pyramids for electron microscopy were trimmed under the preparation microscope so that this area was at the apex of the pyramid and the sections contained both structures whenever possible (*cf. 5, 7, 8*).

RESULTS

Light Microscopic Controls

Normal epidermis.—Figure 2 represents normal skin of the back of the female mouse of RA strain. The IFE is composed chiefly of one layer of nucleated cells of the basal type. Its electron microscopic appearance is presented in (7).

Carbowax 400 orally.—Experiments were made in which undiluted Carbowax 400 without carcinogen was given by means of a stomach tube on successive days 1, 5, and 10. Two doses were used: 0.15 ml, which equaled the dose given as solvent for DMBA, as well as 0.30 ml. The animals were permitted to move freely in their cages; they were not prevented from licking themselves or each other. The hair was cut immediately before biopsy.

In the cutaneous structures no deviations from normal were seen in these experiments, though serial sections were made (fig. 1).

At histologic examination, serial sections from the liver, lungs, stomach, small and large intestine, kidneys, and spleen had a normal appearance.

DMBA orally to immobilized animals.—Mice whose fur was uncut were given 1.6 mg of DMBA in 0.15 ml of undiluted Carbowax 400 by gastric instillation. The animals were immediately put into narrow glass tubes so that all movements, including licking, were prevented. After 24 hours, biopsies were taken from the back. Longer observation periods with animals in the tubes were not possible.

Light microscopic examination of the skin showed identical alterations as experiments with freely moving animals (details given later). The discontinuous localization of the changes necessitated examination of numerous nonconsecutive sections of the same specimens.

Examination under the light microscope of the skin from untreated controls that were similarly held in glass tubes for 24 hours showed no deviation from normal.

Examination under the light microscope of serial sections from the liver, lungs, stomach, small and large intestine, kidneys, and spleen showed no deviation from normal.

DMBA orally to freely moving animals.—DMBA in 0.15 ml of undiluted Carbowax 400 and in 0.25 ml of light paraffin was given once, or on 5 successive days, as gastric instillation. Biopsy specimens from the back were taken after varying intervals at the same time as the whole

internal organs. The individual doses of DMBA varied from 100 γ to 1.6 mg.

Figure 3 is from the back of a mouse given 1.6 mg of DMBA only once. The alterations are apparent. An elongation of the basal-type cells accompanied by a distinct, irregular IFE hyperplasia is conspicuous. The hair canal appears almost empty. Under the polarization microscope the lower poles of the basal cells attaching to the dermoepidermal junction depicted brilliant anisotropic bundles (*cf. 6*). In general, the IFE showed a patchlike, irregular hyperplasia due to development of and increase in compactly built basal-type cells. The number of mitoses was higher than normal. Occasional intercellular bridges were seen also between the basal cells. Within hyperplastic areas, the cells were layered in a disorderly manner. Nuclear and cellular atypia were observed in some areas. The lamellae of the stratum corneum were coarse and irregular. The cutaneous changes were most conspicuous in the pilosebaceous apparatus, and an atrophy of the sebaceous glands was the feature most often seen. There was keratotic plugging within the dilated follicular mouths. In places the hair canal contained deranged or concentric layers of keratin. Musculi arrectores pilorum were often injured. The outer hair sheaths were irregular and exhibited abnormalities in cellular organization related to those in the IFE. Sometimes there was slight perifollicular inflammation. The changes within the pilosebaceous apparatus were independent of the hair follicular cycle.

In experiments in which DMBA was given as a single gastric instillation, cutaneous alterations of the type described were seen to develop as a result of all doses. The changes were already evident 24 hours after the administration. When the dose was 1.6 mg, the alterations were observed more frequently (*cf. 4*). In numerous instances, however, detection of the changes necessitated examination of a considerable number of sections serially cut. When DMBA was given on 5 successive days and the biopsies were taken on the 7th day from the commencement of treatment (figs. 4 and 5), cutaneous changes were seen in all animals and in all sections. The alterations were similar to those developing after a single oral administration of DMBA.

Examination under the light microscope of serial sections from the liver, lungs, stomach, small and large intestine, kidneys, and spleen of mice given a single gastric instillation of 1.6 mg of DMBA revealed no pathologic alterations. On the 7th day, in experiments with 5 successive doses of DMBA, examination of serial sections from the liver, lungs, stomach, small and large intestine, and kidneys revealed very slight changes. In most instances, no alterations from normal could be seen. However, the spleen was sometimes injured; the capsule and trabeculae were thickened, and the malpighian bodies atrophic.

Electron Microscopy

Light microscopy revealed that repeated gastric instillations of DMBA given on successive days highly increased the number of places within the

skin in which the alterations appeared, though their histologic character remained unchanged. To facilitate electron microscopy, most of the work with this technique was performed on animals given DMBA on 5 successive days. Before this, a very large number of electron microscopic sections had been examined from experiments with DMBA administered as a single instillation. The results obtained from both were identical.

The general electron microscopic appearance conformed to that observed in the same material under light microscopy. In addition, it became evident that the ultrastructural alterations were similar to those that developed in the mouse epidermis as a result of local exposure to the same carcinogen in the same resistant mouse strain. To avoid unnecessary repetition of features previously reported in experiments with carcinogens administered locally (*5*), only the main characteristics will be presented here.

Cell surface-to-cell surface contact.—The major difference between the effect on surface contact of local exposure to DMBA and oral exposure was the narrowness or total lack of *intercellular spaces* (figs. 6, 7, 8, 18, and 19). Correspondingly, *intercellular bridges* were few or lacking. The intracytoplasmic fibrillar tufts anchoring to the cytoplasmic faces of the *desmosomes* appeared highly electron dense or were confluenced to each other to form more electron-dense consolidations (figs. 8 and 19). The *dermoepidermal junction* and the *basal attachment devices* (figs. 6 and 7) did not deviate from normal.

Cytoplasm.—Typical was the polymorphic and irregular image of the nucleated cells in hyperplastic areas. At the same time the *nucleo/cytoplasmic ratio* was increased similarly as when the same carcinogen had been administered locally. Here and there, and also in the basal cells, within the irregularly altered IFE and outer root sheaths, there were cells with an electron-dense pattern located paramembranously in the cell periphery. The profile of the alterations was to some extent dependent on the plane of section. Sometimes it appeared as a crown of thornlike pattern (figs. 17 and 18), sometimes as an irregular, electron-dense, whisker-like formation (fig. 6, *arrows*). Under the polarization microscope both formations revealed a brilliant anisotropy indicating the presence of keratin(s). The cytoplasmic matrix of the epidermal cells of the normal mouse back lacks the double membranes of the endoplasmic reticulum, but the whole cytoplasm is scattered with fine granules (*7*). Figures 7, 8, and 19 present alterations of the cytoplasm of the basal cells after oral exposure to DMBA. Almost all the granules have disappeared; instead there are delicate, more electron-dense, parallel interfaces [*cf.* figs. 3 and 9 of the same places in normal basal cells (*7*)]. This increased lamellar density appeared particularly in the cell periphery.

The *mitochondria* showed a variety of distinct changes identical with those seen in altered epidermal cells after local exposure of the skin to the same carcinogen (*5*). Figures 9 to 16 illustrate several different varieties of mitochondrial changes in several animals. Conspicuous was the fact that a given cell could have mitochondria possessing a normal ultrastruc-

ture (fig. 11), whereas those in the neighboring cells were altered. The typical feature was an intracristal accumulation of 1 to 3 round or oval, electron-dense inclusion bodies that were surrounded by only one membranous profile (figs. 13 to 16); otherwise the double-membranous behavior of the internal cristae was preserved. Sometimes the globules were so big that they filled the whole mitochondrial volume. In other mitochondria the matrix appeared diffusely blurred, and the internal cristae were translucent or invisible. Virus-like particles were not seen.

Stratum corneum.—As was to be expected from the light microscopy of the same material, electron microscopy revealed severe disturbances in the stratum corneum and hair. The keratogenous lamellae were uneven and their amount varied from one place to another. Furthermore, the keratin contained various types and grades of immature degradation products and cellular remnants (figs. 20 and 21).

The degree and character of the alterations in various ultrastructures of mouse epidermis that had developed after gastric instillation of DMBA were independent of the phase of the hair follicular cycle as were the alterations developing as a result of local exposure to the same carcinogen on the same resistant mouse strain (*5*).

DISCUSSION

When compared with the ultrastructural changes developing in the epidermis of the back of the mouse of a resistant strain as a result of local exposure to 7,12-dimethylbenz[*a*]anthracene (*5*), gastric instillation of the same carcinogen to mice of the same strain brought about identical changes in the same cutaneous structures. The alterations were: (*a*) In light microscopy, different abnormalities in cell surface-to-cell surface contact indicate disturbances in detachment of epidermal cells ["stickiness" of the cells that were dependent on development of cable-like adhesions of anisotropic bundles, particularly between the basal cells and dermo-epidermal junction (*6*)]; (*b*) development of new profiles, *e.g.*, a crown of thornlike pattern in the paramembranous cytoplasm also in the basal cells; (*c*) disturbances in keratinization and appearance of immature degradation products in places where they are not seen normally; (*d*) accumulation of electron-dense globular inclusion bodies intracristally within the internal mitochondrial membranes even in the basal cells, and (*e*) a characteristic variability in degree and quality of the alterations from one place in the skin to another.

That the alterations listed did not depend on irrelevant factors such as external contamination with the carcinogen was established by a series of special experiments in which identical results were obtained. The changes are therefore to be considered specific of the effect of the DMBA. The following chain of facts favors this statement: (*a*) The experiments were repeated and identical alterations observed; (*b*) the changes developed *intra vitam*—they were evident in light microscopy, and polarization

microscopy revealed purposefully arranged anisotropic features that were never seen in normal controls with experiments with resistant mouse strains, but were characteristic of the effect of locally applied DMBA (*5*); (*c*) the alterations were not brought about by the solvent, since a 20 times higher total dose of Carbowax 400 failed to cause any changes in the epidermis, and because alterations were also obtained with DMBA in light paraffin; (*d*) the changes could not be regarded simply as indicative of conditions such as a nonspecific epidermal response to a hypothetical generalized "stress," because the changes observed in light microscopy contained morphologic criteria generally accepted in classical pathology as characteristic of the effect of a carcinogen; (*e*) nor could there be any question of a general toxic effect during the relatively short experimental period (from 24 hours to 7 days), as the light microscopic examination of serial sections of the liver, lungs, stomach, small and large intestine, and kidneys did not reveal any pathologic alterations; only the spleen of some animals after treatment with successive high doses of DMBA showed atrophic features; (*f*) the alterations of the epidermis seen in light and electron microscopy were independent of the dose of DMBA—nor were they affected by the hair follicular cycle; (*g*) it is relevant to state that the keratinization in the stratum corneum was delayed because the keratin lamellae contained immature degradation products and various cellular remnants, as well as high amounts of sulfhydryl, whereas the concentration of disulfide was almost zero (*cf. 6*); and (*h*) the experimental animals were females with healthy back skin.

The mitochondrial changes deserve special comment. No destruction, general irregularity, decomposition, or dissociation was seen in any of the ultrastructural profiles of the mitochondria as may well be the case in nonspecifically injured, dying epidermal cells. On the contrary, the alterations possessed a recurring feature: The electron-dense globules were always located intracristally within the internal membranes instead of being free within the matrix (figs. 13 to 16) and were surrounded only by a single membrane. Only when the globules grew large enough could they fill the whole mitochondrial cavity. Because identical mitochondrial changes have been seen to develop in the epidermis of this tumor-resistant strain as a result of both local (*5*) and oral administration of DMBA, as well as after treatment of the skin of highly tumor-susceptible Swiss mice with Tween 60 without any carcinogen (*8*), these alterations must possess a causal connection with the carcinogenetic process. Furthermore, because the mitochondrial globules were always located intracristally, their presence reduced the surface area of the internal double membranes. This decrease in surface area may cause an impaired cellular respiration with its consequences [*cf.* results and conclusions in (*5*, *8*)]. In this connection it is relevant to refer to a recent observation concerning mitochondrial alterations of an *Acanthamoeba:* Similar intracristal accumulation of globular electron-dense inclusion bodies in the mitochondria occurs at encystation of the ameba at a time in the life cycle when the respiratory activity is reduced to a minimum (*9*). It will be very interesting to

compare the alterations in mitochondria in experiments with DMBA with those in certain tissues of hibernating mammals (in preparation).

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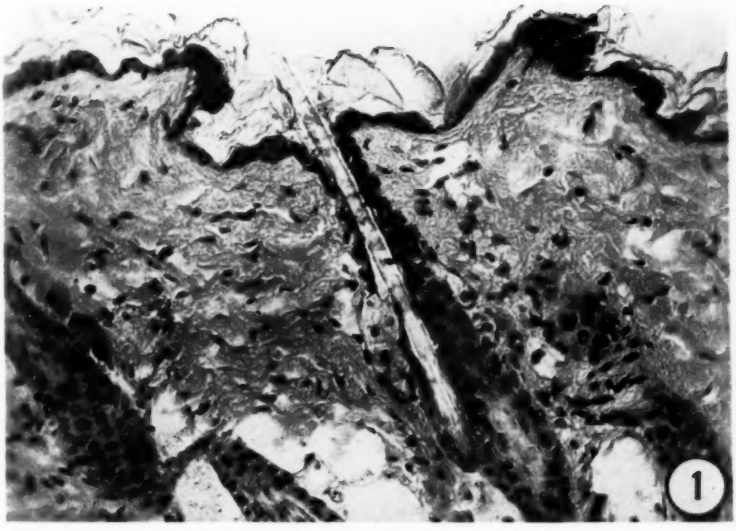

FIGURE 1.—Mouse #22358. Five gastric instillations, on successive days, of 0.15 ml of undiluted Carbowax 400 without DMBA; 7th day: no deviation from normal; all structures preserved. Hair follicular cycle: catagen. × 270

PLATE 129

FIGURE 2.—Mouse #14018. Normal skin and hair follicular mouth of back of untreated strain RA mouse. Hair follicular cycle: telogen. × 800

FIGURE 3.—Mouse #14755; 1.6 mg of DMBA in 0.15 ml of Carbowax 400 once intragastrically, 2d day: irregular, distinct hyperplasia of IFE and root sheath. Distinct intercellular spaces between basal cells that are elongated and in "sticky" connection with dermoepidermal junction. Elongated lower pole of these cells depicts plenty of anisotropic fibrils and cable-like bundles when examined in polarized light (*cf.* 6). Hair destroyed. Hair follicular cycle: telogen. × 800

FIGURE 4.—Mouse #21069; 1.6 mg of DMBA in 0.15 ml of Carbowax 400, intragastrically on 5 successive days, 7th day: IFE and hair-root sheath are severely deranged. Hair canal dilated, containing irregular layers of keratogenous material. Hair follicular cycle: anagen. × 800

FIGURE 5.—Mouse #21060; 1.6 mg of DMBA in 0.15 ml of Carbowax 400, intragastrically on 5 successive days, 7th day: alterations similar to those in figure 4, independent of hair growth phase. Hair follicular cycle: telogen. × 800

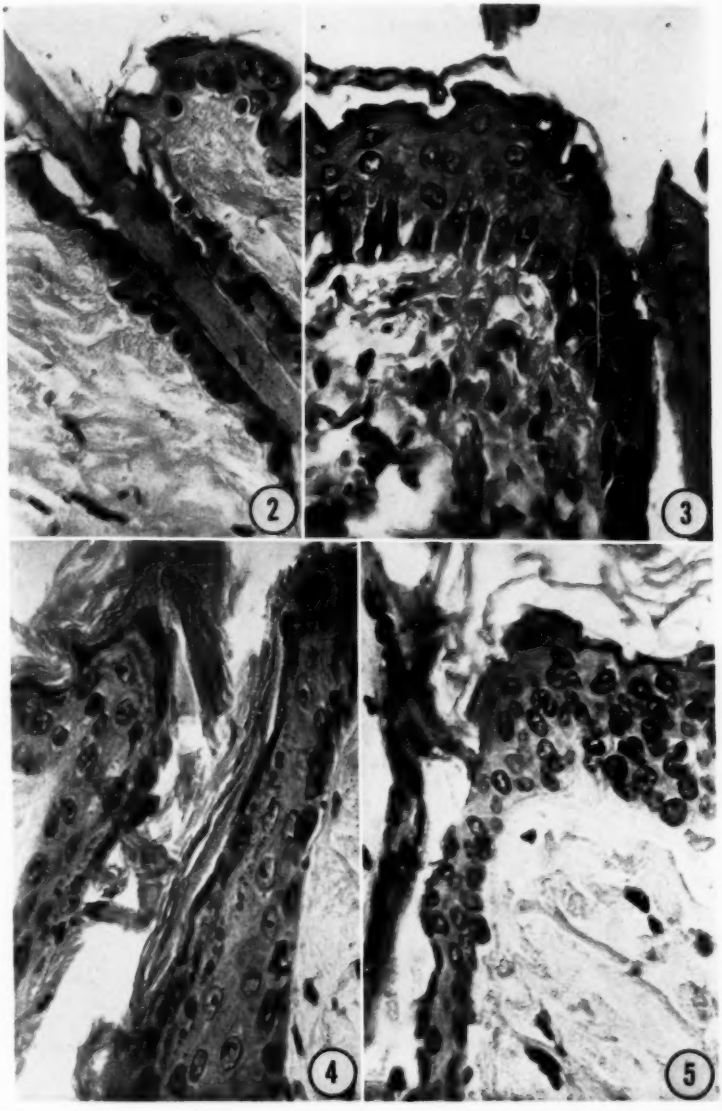

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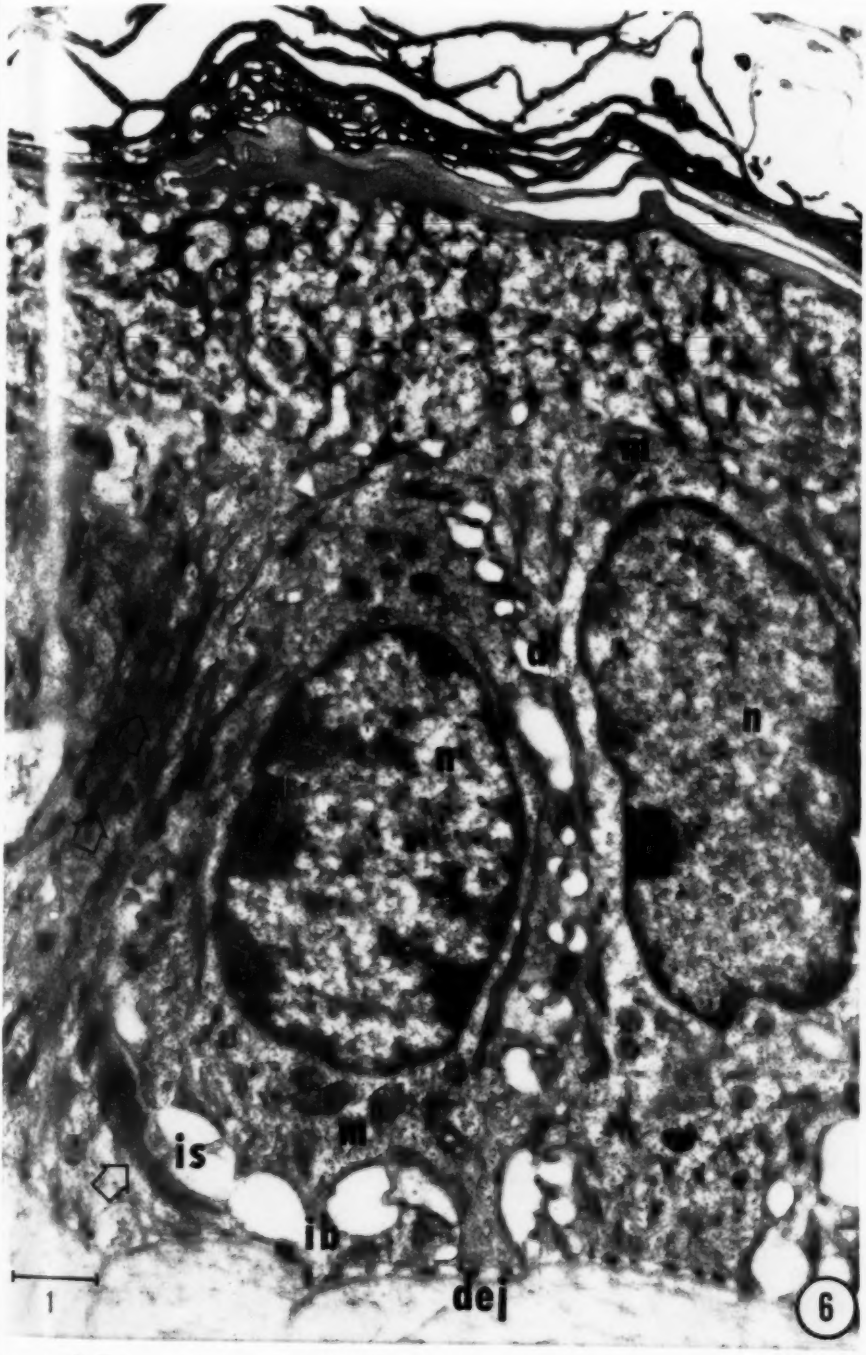
PLATE 130

The following designations on electron micrographs indicate cellular components: bad, basal attachment devices; cb, cell boundaries; col, collagen bundles; d, desmosomes; dej, dermoepidermal junction; ib, intercellular bridges; is, intercellular spaces; m, mitochondria; n, nuclei.

FIGURE 6.—Same animal as in figure 5: 1.6 mg of DMBA in 0.15 ml of Carbowax 400, intragastrically on 5 successive days, 7th day. Electron micrograph of altered IFE. Distinct spaces both between neighboring basal cells and between former and dermoepidermal junction. Abundant electron-dense fibrous profiles arranged as bundles (*arrows*) that possess brilliant anisotropy in polarized light. Mitochondria almost normal. Hair follicular cycle: telogen. × 13,400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is
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dej
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PLATE 131

FIGURE 7.—Mouse #21061; 1.6 mg of DMBA in 0.15 ml of Carbowax 400, intragastrically on 5 successive days, 7th day: electron micrograph from basal portions of two adjacent basal cells illustrating alterations of cytoplasmic ultrastructure characteristic of effect of exposure to DMBA. Transformation of originally finely granulated cytoplasm to delicate profiles that appear in sagittal sections as parallel interfaces [*cf.* finely scattered cytoplasm of exactly same region of normal IFE in fig. 3 in (7)]. No virus-like particles. Hair follicular cycle: anagen. × 63,500

n
m
m
bad
dej
1
7

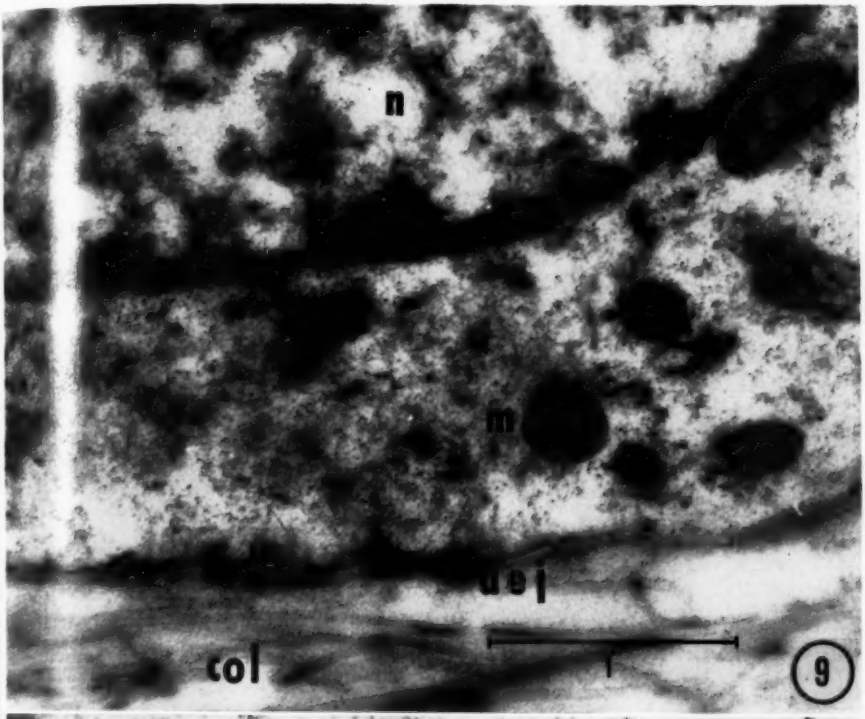
PLATE 132

FIGURE 8.—Same animal as in figure 7: electron micrograph from middle level of nuclei of altered basal cells, illustrating transformation of originally finely scattered cytoplasm into delicate, parallel interfaces [*cf.* finely granulated cytoplasm of same region and level of normal IFE in fig. 9 in (7)]. × 60,000

d
n
n
b
8

PLATE 133

FIGURES 9 to 12.—Mouse #21068, hair follicular cycle: telogen (figs. 9 and 12); #21064, hair follicular cycle: telogen (fig. 10); and #21061, hair follicular cycle: anagen (fig. 11). Electron micrographs from *3 different animals*, illustrating mitochondrial alterations caused by gastric instillation of 1.6 mg of DMBA in 0.15 ml of Carbowax 400, on 5 successive days, 7th day. Some mitochondria appear normal (fig. 11); in others matrix appears diffusely electron dense (figs. 9, 10, and 12). (*Cf.* alterations in figs. 13 to 16.) Figure 9: × 37,500; figure 10: × 60,500; figure 11: × 95,000; figure 12: × 33,500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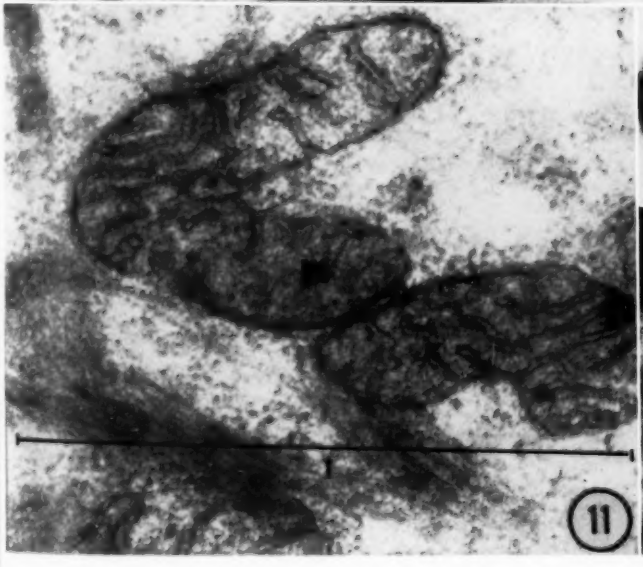
11

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m
10

m
12

PLATE 134

FIGURES 13 to 16.—Mouse #21061 (figs. 13 to 15); #21069 (fig. 16), hair follicular cycle in both: anagen. Electron micrographs from *4 different places from 2 animals*, illustrating mitochondrial alterations within epidermal cells caused by gastric instillation of 1.6 mg of DMBA in 0.15 ml of Carbowax 400, on 5 successive days, 7th day. Almost all mitochondria contain 1 to 3 round or globular, electron-dense inclusion bodies. These osmiophilic globules lie intracristally; internal cristae then continue as double membranes onto outer mitochondrial double envelope. These alterations are identical with those resulting from local application of DMBA (*5*). Figure 13: × 73,500; figure 14: × 52,000; figure 15: × 52,000; figure 16: × 52,000

FIGURE 17.—Mouse #21060. Same treatment as in figures 13 to 16. Electron micrograph from altered epidermal basal cell with electron-dense crown of thornlike profile at cell periphery (*arrows*) that depicts brilliant anisotrophy when examined in polarized light. Hair follicular cycle: telogen. × 11,500

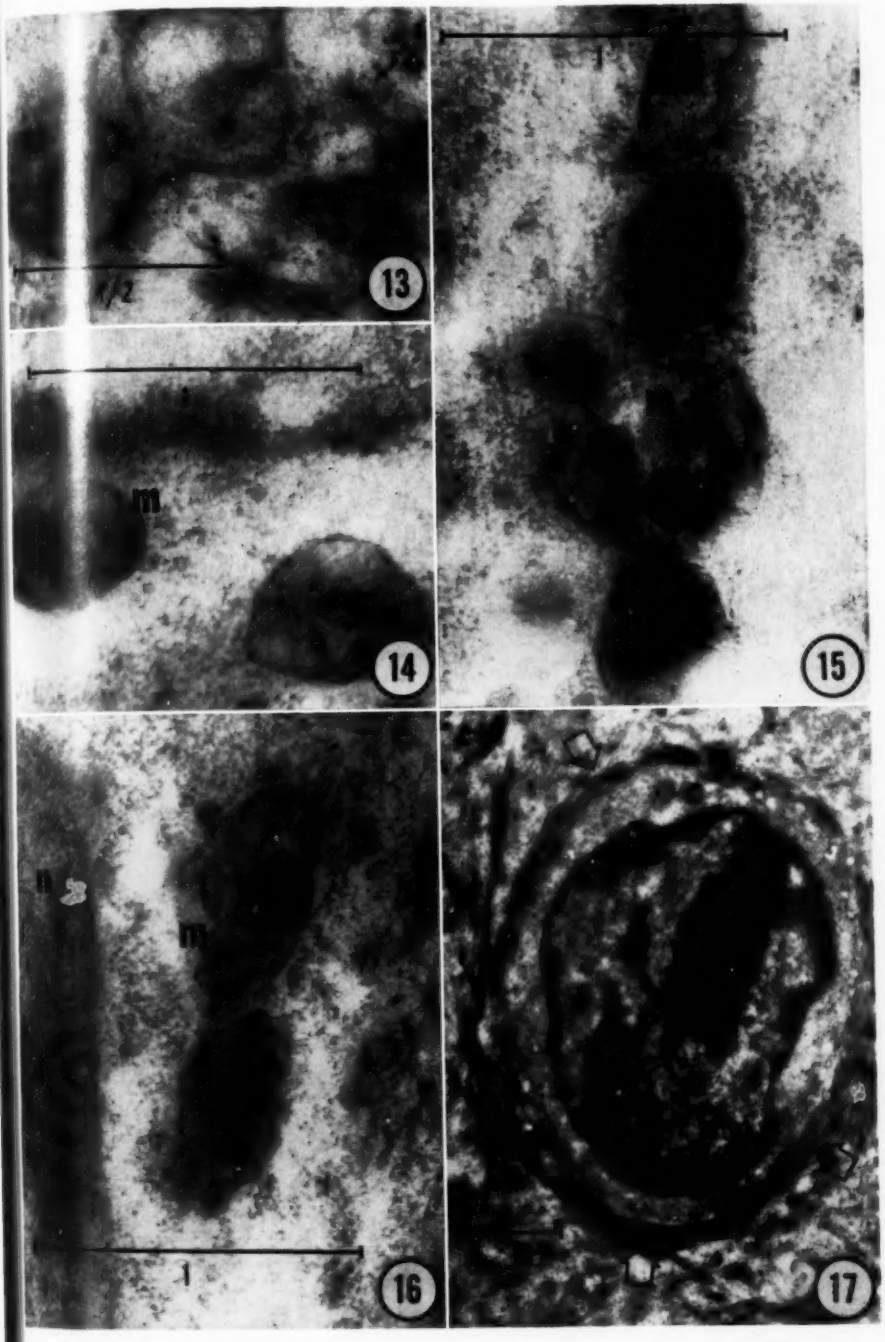
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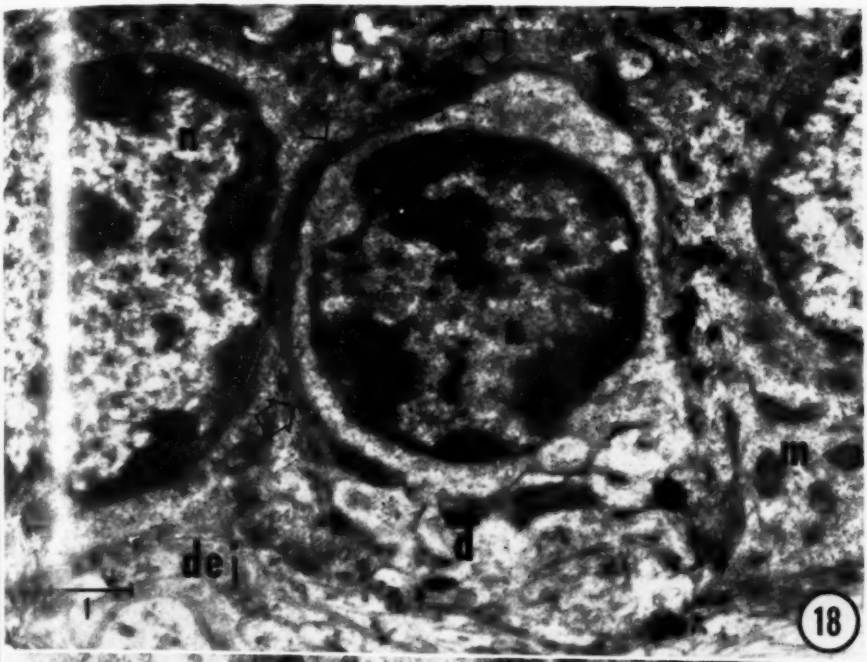
PLATE 135

FIGURE 18.—Mouse #21061; 1.6 mg of DMBA in 0.15 ml of Carbowax 400, on 5 successive days, 7th day; electron micrograph from altered basal cell (*center*), illustrating existence of electron-dense crown of thornlike profile at cytoplasmic periphery (*arrows*) touching, at places, desmosomal organelles. Hair follicular cycle: anagen. × 12,500

FIGURE 19.—Mouse #21062. Same treatment as in figure 18. Electron micrograph from slightly oblique sagittal section from neighboring basal cells. Cell boundaries intimately follow each other. Paramembranously (*arrows*) electron-dense interfaces—at these places amount of cytoplasmic granules decreased or nil. Hair follicular cycle: anagen. × 75,000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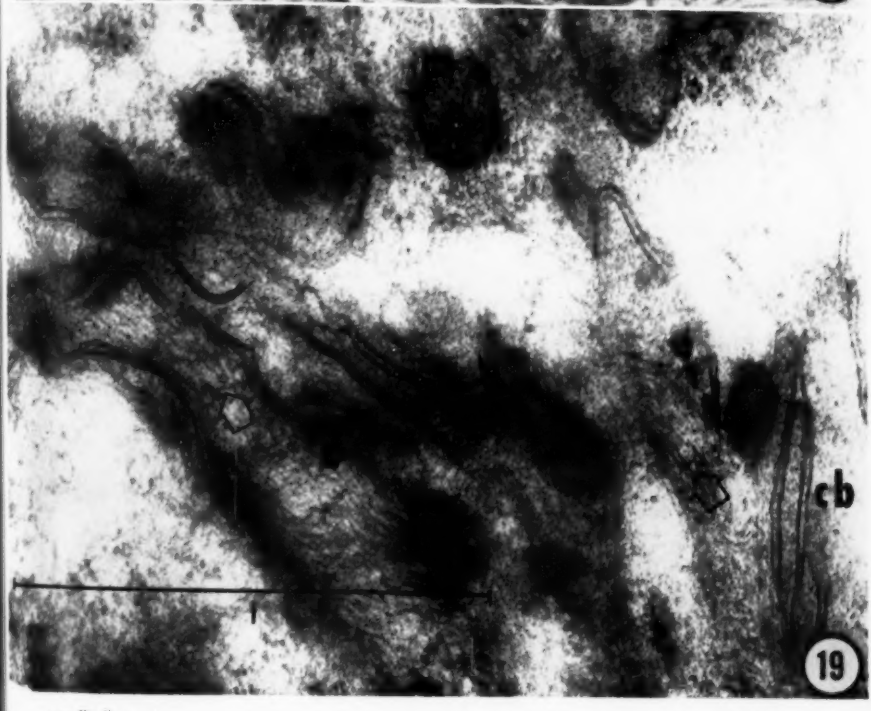
cb
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19

PLATE 136

FIGURE 20.—Mouse #21074; 1.6 mg of DMBA in 0.15 ml of Carbowax 400, on 5 successive days, 7th day: electron micrograph from superficial IFE and stratum corneum, which demonstrates disturbances in keratinization, existence of various cellular remnants, and immature degradation products within keratogenous lamellae. Hair follicular cycle: telogen. × 40,500

FIGURE 21.—Mouse #14750; 1.6 mg of DMBA in 0.15 ml of Carbowax 400 as a single gastric instillation, 2d day: electron micrograph from stratum corneum, which shows that even a single oral administration of carcinogen severely disturbs keratinization and causes development of immature degradation products within stratum corneum. Hair follicular cycle: telogen. × 12,500

1
20
1
21

Morphological Evidence for the Association of Virus Particles With the Pancreatic Acinar Cells of the Chick [1]

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SUMMARY

Particles, apparently morphologically identical to virus particles described in various avian systems, "budded" or formed from the apical plasma membrane of pancreatic acinar cells in 5 of 12 chicks and embryos chosen at random from "normal" colonies. The particles were composed of two concentric, dense membranes with a centrally located, dense nucleoid, and their diameter was approximately 800 A. The lungs, livers, kidneys, hearts, spleens, and intestines of these 5 chicks contained morphologically identical particles in intercellular spaces, intracytoplasmic vesicles, and vacuoles. When particles were not associated with the pancreas of several birds, they could not be found in other organs. The significance of this observation in relation to avian neoplasms and normal tissues is discussed.—J. Nat. Cancer Inst. 26: 1011–1039, 1961.

MANY INVESTIGATORS have associated virus particles, approximately 800 A in diameter, containing a dense nucleoid of about 350 A in diameter [class C virus particles of Bernhard (*1*)] with diseases in the domestic fowl. Pertinent to the field of avian neoplasia are the studies concerning the Rous sarcoma (*2–9*), visceral lymphomatosis (*10–12*), myeloblastosis and erythroblastosis (*13–28*), myxosarcoma de Fujinami (*29*), endothelioma of Murray-Begg (*30*), and a chemically induced chicken sarcoma (*31*), in which several particles were described, including a class C particle. Structures apparently identical morphologically to those previously described have been observed in certain "normal" tissues of the domestic fowl (*27, 28, 32*). Most reports describe the majority of the class C particles as extracellular or enclosed in intracytoplasmic, membrane-bound vesicles. The various avian agents associated with neoplasia have not been differentiated on a morphological basis, and some are immunologically similar (*33*). Whether the particles observed within the vesicles, vacuoles, and "gray-bodies" (*26*) represent virus-particle formation has not been completely resolved. Particles in these regions of the

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cell may be results of phagocytosis, degeneration, lysis, or "infecting" processes. Other information concerning particle formation, such as that in influenza virus (*34*), the milk agent (*35*, *36*), particles in a mouse salivary-gland tumor (*37*), mouse leukemia agent (*38*), the Friend agent (*39*), and plasma-cell tumors (*40*), suggests that particles partially composed of membrane components sometimes originate in association with a membrane system of the cell and exhibit a characteristic sequence of formative stages. Similar types of "budding" processes, which can account for the relatively large numbers of particles, have not been convincingly described or demonstrated in chicken tissue, either normal or neoplastic. Isolated and infrequent reference has been made to particles passing through the plasma membrane or "budding" from available membrane systems (*15*, *28*, *32*, *41*).

While the various avian agents, such as Rous, lymphomatosis, myeloblastosis, and erythroblastosis, are widely distributed and have long been the objects of intensive research, relatively little is known of their sites and mode of formation. This report presents preliminary evidence indicating a possible site and a pattern of formation of particles that possess ultrastructural features identical to those of many known avian viral agents.

MATERIALS AND METHODS

Several chicks in this study were obtained by the National Cancer Institute from a colony of New Hampshire Reds. Some embryos (White Leghorn) were received from Cobb's Farm of Pedigreed Chickens in Littleton, Massachusetts. Various chicks and embryos were chosen at random from the "normal" colonies. At the time the various tissues were taken for fixation, all chicks appeared healthy. Seven 3-day-old, one 15-day-old, and three 8-day-old chicks, and one 7-day incubated embryo were used. Tissues were prepared for electron microscopy in the following manner: Chicks were decapitated and viscera exposed immediately; splenic regions of the pancreas and other tissues were diced in chrome osmium (*42*), fixed for 1 hour at 5° C, washed with 10 percent neutral formalin at 5° C, post-fixed for another hour in this solution (*43*), and rapidly passed through graded percentages of ethanol for dehydration. The tissues were infiltrated with and embedded in a mixture of *n*-butyl and ethyl methacrylate 3:1. The reaction was catalyzed with 0.25 percent benzoyl peroxide. The tissues and prepolymerized plastic [3] were put in capsules and allowed to polymerize completely in an 80° C oven for 24 hours. Thin sections were cut from the blocks with a diamond knife on a Porter-Blum (Servall) microtome, picked up on Formvar-coated grids from a 25 to 30 percent acetone-in-water filled

[3] The inhibitor was removed from the monomers with NaOH, which was washed free from the plastic with several changes of water, and the plastic was dehydrated by storing over Linde molecular sieves. The plastic was cleaned by shaking with activated charcoal before final filtration and dehydration.

trough, and stained routinely (*44*). The electron micrographs were made with an RCA EMU-2-C, fitted with a 15 mil condenser aperture (Canalco[4]) and 30 μ or 50 μ platinum objective apertures (Burger).[5] Exposures were made on Kodak, contrast, antiabrasion photographic plates at original magnifications of approximately 8,000 to 19,000 and enlarged optically to the desired number of diameters.

OBSERVATIONS

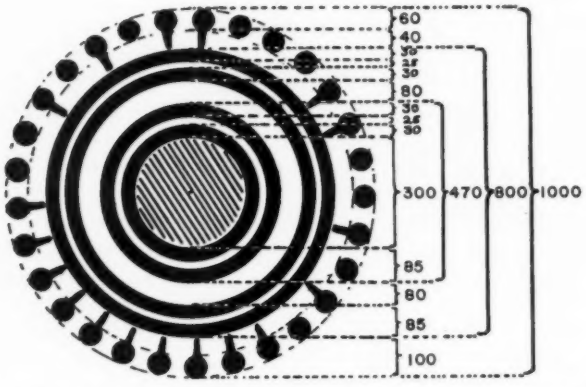
The general histological pattern of the chick pancreas is similar to that of other animals (*45–50*). The acinar cells are generally pyramidal and possess a prominent nucleus (approximately 5 μ) with a large (1.3 μ) nucleolus, elongated mitochondria, a rich ergastoplasm, and usually a large complement of zymogen granules (0.9–1.4 μ). A distinct Golgi region usually lies between the nucleus and the apical region of an acinar cell. Microvilli, terminal bars, and occasionally desmosomes are associated with the plasma membrane in the apical region of the cell. Other micrographs of this tissue are presented elsewhere (*44*).

Particles, apparently morphologically identical to previously described avian virus particles, were first noted in pancreatic cells of an 8-day-old chick. A budding process occurred only in the apical region of the acinar cells (figs. 6 and 7), where practically every acinus observed in thin section contained at least a few particles. Figures 1, 2, 6, 7, 10, 11, 13, 16, 17, and 18 illustrate particles presumably in early stages of a budding process. Figures 2, 4, 5, 7, 13, 16, and 17 show several particles whose nucleoids are somewhat more dense. Figures 2, 4, 5, 9, and 15 exhibit particles characteristic of the avian agents, and all possess dense nucleoids. Some of the particles "bud" in close proximity to the cell mass (figs. 1, 6, 10, and 11), while others are attached to the cell by elongated stalk ends (figs. 2, 7, 14, 16, and 17). Evidence of virus particles, viroplasm, "gray bodies" (*27*), or virus formation by the "budding" process was not observed in the nucleoplasm or within the cytoplasm of the acinar cells. A ribonucleoprotein (RNP), particle-free space usually underlies the plasma membrane of the acinar cells. This space consistently is in the limited regions where the particles are forming. This zone of moderately dense filamentous material is similar to the terminal web described in intestinal epithelium (*52*). Cells of a mouse salivary-gland tumor showed a similar region (*38*).

When well-preserved and in presumably early stages of the budding process, the particles consist of 5 dense, concentric rings (figs. 4, 5, 6, and 7). These may represent 2 membranes, each composed of 2 layers of approximately 30 A. These layers are separated by a light space of approximately 25 A. A fifth outer dense ring, that does not appear to be of

[4] Canal Industrial Corp., 4951 St. Elmo Ave., Bethesda, Md.
[5] Burger, G. F., 20 St. Martin's Road, Merchantville 8, N.J.

membrane material, surrounds the particles. This ring is sometimes seen when the particles are still attached to the plasma membrane (figs. 6 and 7). The small, dense particles apparently composing this ring are not associated with the plasma membrane where particles are not forming. It adds approximately 200 A to the total diameter of the particles that, measured from outer membranes, is approximately 800 A, corresponding to the figures given previously (*1*, *17*, *28*) for virus particles in other domestic-fowl tissues; thus, the total diameter is about 1000 A. Text-figure 1 is a diagrammatic representation of particle morphology derived from detailed study and measurement of several particles in figure 5. It illustrates the calculated spacings of the various components in typical particles.



TEXT-FIGURE 1.—Schematic drawing of average particle from figure 5. Dimensions are in angstroms. Approximately × 830,000

Mature particles frequently were in the pancreas of the 8-day-old chick, in intercellular spaces, between parenchymal cells (fig. 3), in the basement membrane, and near the endothelial linings of the blood capillaries, but not in the cytoplasm or large vesicles or vacuoles of the acinar cells. Rarely, a single particle appeared to be in a small membrane-bound vesicle in an acinar cell, but always near the periphery of the cell (fig. 2). It is quite possible that these represent a particle lying in an oblique section of a small depression in the plasma membrane. No more than one particle similar to those observed in the various normal and neoplastic tissues previously described for the avian system was observed in vesicles or vacuoles of the acinar cells.

Other embryonic and posthatched pancreases were also studied. In the 12 chicks and embryos examined, 5 possessed this entity, the morphology of which resembles avian viruses. Figure 8 illustrates the particles observed near the base of an acinar cell in a 7-day-incubated embryo. Particles were also "budding" from the apical plasma membrane of the acinar cells. Figure 19 illustrates an acinar lumen of a 3-day-old

chick. Particles are in a packed pattern similar to that obtained from pellets of concentrated virus particles from experimental procedures. Figure 18 shows several "budding" particles in a 3-day-old chick.

In several cases, pancreas, liver, lung, kidney, spleen, small intestine, and heart were prepared for electron microscopy. In chicks where particles were demonstrated to "bud" in the pancreas, the other tissues examined contained "mature" particles. The particles were located in intracytoplasmic vesicles and vacuoles and in intercellular spaces. Figure 20 is a micrograph of a section near a pulmonary alveolus from a 3-day-old chick with a pancreas positive for particle formation. The particles were in close association with the epithelial, endothelial, connective-tissue, and basement-membrane regions, but were not free in the alveolar lumina of the lung. Figure 21 exhibits particles associated with renal tissue in a 3-day-old chick. This section represents a portion of a distal convoluted tubule. Particles were occasionally free in the lumina of both the proximal and distal convoluted tubules. When particles were in the process of "budding" in the pancreas, the heart, liver, small intestine, and spleen also contained particles in small vesicles, vacuoles, and intercellular spaces. No evidence indicated that particles "bud" from the membranes of cells in any other organ examined. In the 7 chicks whose pancreases were not associated with particles, no particles were found in any other tissues examined.

Particles in tissues at a distance from the pancreas do not appear to have as crisp or sharp a profile. The membranes frequently look damaged, swollen, wrinkled, and distorted. It is difficult to resolve the 30-A unit layers of the two membranes associated with the particles containing a dense nucleoid. Figures 3, 8, 9, 15, 20, and 21 illustrate particles whose membranes appear separated and distorted. The various units are not as well resolved as when the particles are apparently in early stages of the "budding" process.

DISCUSSION

No existing evidence derived from bioassay indicates that these particles of pancreatic origin represent infectious virus particles, but they do possess morphological characteristics which strongly suggest that they are related to infective agents: The particles are composed of two concentric membranes, enclosing, when mature, centrally located nucleoids [class C particles of Bernhard (*1*)]; the dense nucleoids have an affinity for lead subacetate stain, as do the RNP particles, nucleolar components, and the nucleoids of known RNA-containing virus particles; the particles have been shown "budding" from the apical plasma membranes of the pancreatic acinar cells in 40 percent of the chicks examined; the structure of these entities is apparently identical to the particles associated with visceral lymphomatosis, erythroblastosis, myeloblastosis, and Rous sarcoma; the particles were observed to form in the pancreas of an embryo,

as well as in posthatched chicks; when particles could be demonstrated to be "forming" in the pancreases of "normal" chicks, all the other tissues examined (lung, liver, kidney, spleen, small intestine, and heart) contained morphologically identical particles in vesicles, vacuoles, and in intercellular spaces.

From observations on many sections, the following scheme of particle formation is suggested: The outer plasma membrane buds out and a secondary or inner membrane originates beneath it; a general thickening of the inner membrane ensues; as the particle is detached from the cell by being pinched off at the base, the thickening process continues until the space is filled within the inner membrane. This dense central region constitutes the nucleoid of the mature, morphologically defined particle. It is inferred that the figures presented do indeed represent formative stages. The literature on other viral systems suggests that a similar budding process is frequently involved in the production of virus particles associated with one or more available cell membrane systems. The great morphological enigma in this, as well as other "budding" processes, is: Where are the particle components in the cell before they are "organized" at the membrane surfaces of the cell?

The morphological observation of formation of particles similar to those in avian-virus tumors suggests certain possible approaches to many important and interesting problems. Certain speculations and correlations warrant a discussion at this time.

The major problem is where this particular particle type fits in, if it is in any way related, with other known avian viral agents. If the various avian viruses are discrete and distinct entities, then which one does this pancreatic particle represent? Yet, if the various avian agents are very closely related, the possible significance of this particle type is multiplied [*see* Burmester for Review (*11*)]. The possibility exists that this particle could be associated with an undescribed "disease" of chickens and represents a "new" virus particle. At present, however, it most closely resembles, morphologically, the particles associated with various neoplastic and other "normal" tissues in the chick (*e.g.*, visceral lymphomatosis).

The natural transmission of certain anomalies could be envisaged in the following manner: "Viruses" originating with a coat of pancreatic plasma-cell membrane probably have a high resistance to the proteolytic enzymes of the gastrointestinal tract. The membrane may serve as a protection against external influences. A similar function has been suggested for membranes in association with the compartmentalization of enzymes or enzyme precursors in the pancreatic cells (*53*). Since the pancreatic duct joins the lumen of the small intestine, the particles may have access to the gastrointestinal tract and be carried through fecal material. When particles were in the pancreas of the chick, they were also seen in the kidney, lung, and liver. Particles may traverse the epithelial linings of these organs and pass out of the birds via the cloaca, respiratory tract, and upper oral tract.

Particles in various chick tissues not near the pancreas frequently have distorted membranes; it is more difficult to resolve the various units of their structure. These disrupted profiles may be results of environmental exposure and could possibly serve as a criterion for detecting matured or older particles. Particles observed in most of the "diseased" tissues, vesicles, vacuoles, and intercellular spaces, give this appearance. Micrographs of particles prepared by differential centrifugation often present a similar picture.

Production of particles by pancreases in embryos tends to confirm the fact that viruses may be transmitted through the egg (*11*). At least by 7 days of incubation the pancreas has been shown to be capable of producing particles.

This investigation strongly suggests that the morphologically similar particles observed in various tissues other than the pancreas are probably of pancreatic origin. After extensive observations of thin sections which contain particles, it is highly possible that nearly every cell in the chick must at one time be confronted with one of these particles. Practically every disease known to be associated with particles in the domestic fowl involves a cell type which is highly active in the natural state in phagocytotic-like or pinocytotic-like transport processes.

The observation of an outer ring on some of the particles forming in the pancreatic lumen is only of speculative interest at the present. Since there is some specificity of the lead stains in this region, further study is indicated. It is conceivable that these dense bodies surrounding the particles with an estimated volume of approximately 100 to 600 $m\mu^3$ might represent zymogen complexes aggregating about the particles in the lumina of the pancreas. This pattern is not associated with regions of the apical plasma membrane where particles are not forming, however, and thus a more interesting speculation must be considered: The fifth outer ring might represent an antibody reaction. Rifkind and Morgan (*54*), by labeling antibodies (*55*) to influenza virus with ferritin, have demonstrated ferritin micelles arranged about the virus particles at a distance comparable to that measured for the fifth ring in these figures.

The involvement of particles with the pancreas is the first strong morphological evidence *in vivo* of "virus-particle" formation in an avian system. Elucidation of the mode and site of particle formation *in vivo* could conceivably be more definitive than comparable results *in vitro* and might well differ from them. In a tissue-culture chamber there are no other tissues present; therefore, probably no conceivable antibody feedback mechanism could be working, as there probably is in the natural state. The possibility exists that the tissue giving rise to particles in any particular system and those tissues which become associated with a disease may be entirely different.

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PLATE 137

FIGURE 1.—Portion of acinar lumen in 8-day-old chick. *Note* mitochondria (M), zymogen granules (Z), terminal bars (TB), ergastoplasm (ER), and lumen of acinus (AL). Ribonucleoprotein particles (RNP) are associated with endoplasmic reticulum and occur free in cytoplasm. RNP-particle-free space (PFS) underlies budding "virus particle" (VP). Stained with lead acetate and ammonia vapors. Approximately × 53,000

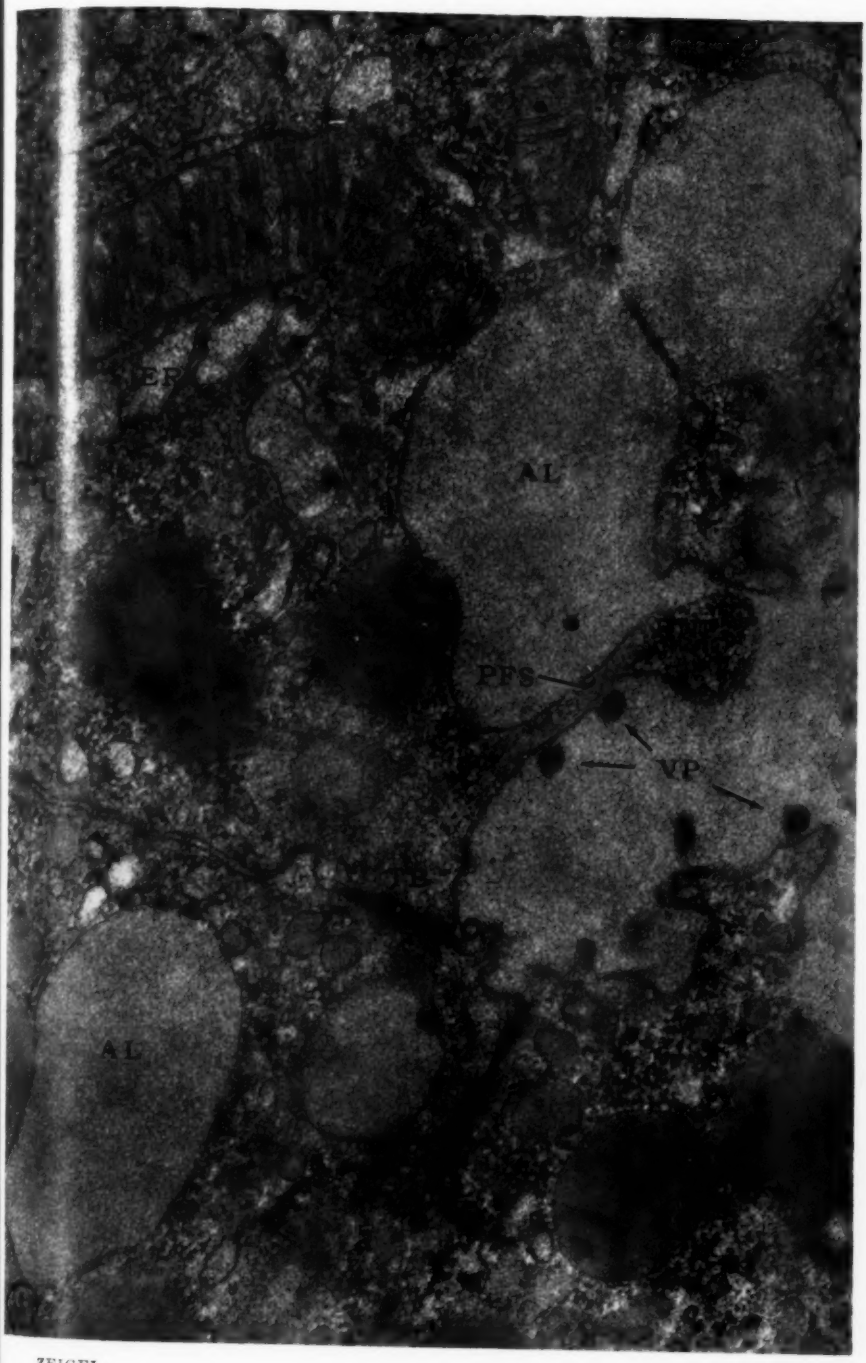

ER
AL
PPS
VP
AL

PLATE 138

FIGURE 2.—Acinar lumen in pancreas of 8-day-old chick which exhibits several "virus particles" budding from apical plasma membrane. Zymogen granules (Z), mitochondria (M), terminal bars (TB), and ergastoplasm (ER) are present in pancreatic cells. "Virus particles" 1, 2, 3, and 4 are attached to cell by cytoplasmic connections. In particles 1, 2, and 3, inner membrane is not yet completed. Particles 5, 6, and 7 are mature particles containing centrally located dense nucleoids. Particles 6 and 7 are perhaps lying in slight depressions in plasma membrane but might be interpreted as intracellular. They are, however, surrounded by what appears to be apical plasma membrane. Stained with Palay's modification of lead-hydroxide technique. Approximately × 53,000

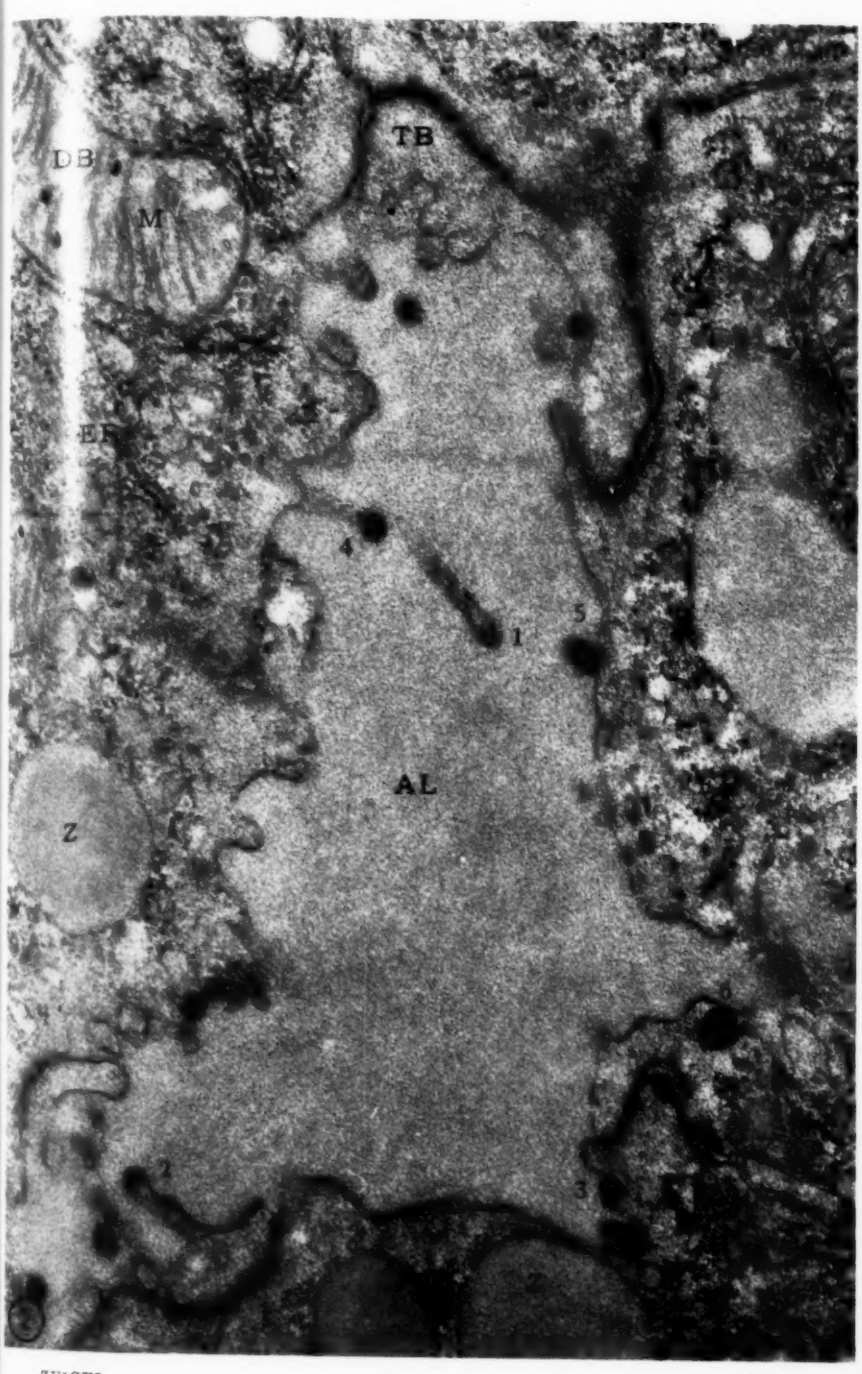

DB
TB
M
ER
4
1
5
AL
Z
2
3

PLATE 139

FIGURE 3.—Typical mature particles may be observed in intercellular space between 2 adjacent acinar cells in pancreas. Mitochondria (M), with intramitochondrial bodies (DB) are visible. The endoplasmic reticulum and associated ribonucleoprotein particles (ergastoplasm, ER) are abundant in lateral and basal regions of these cells. No stain. Approximately × 81,000

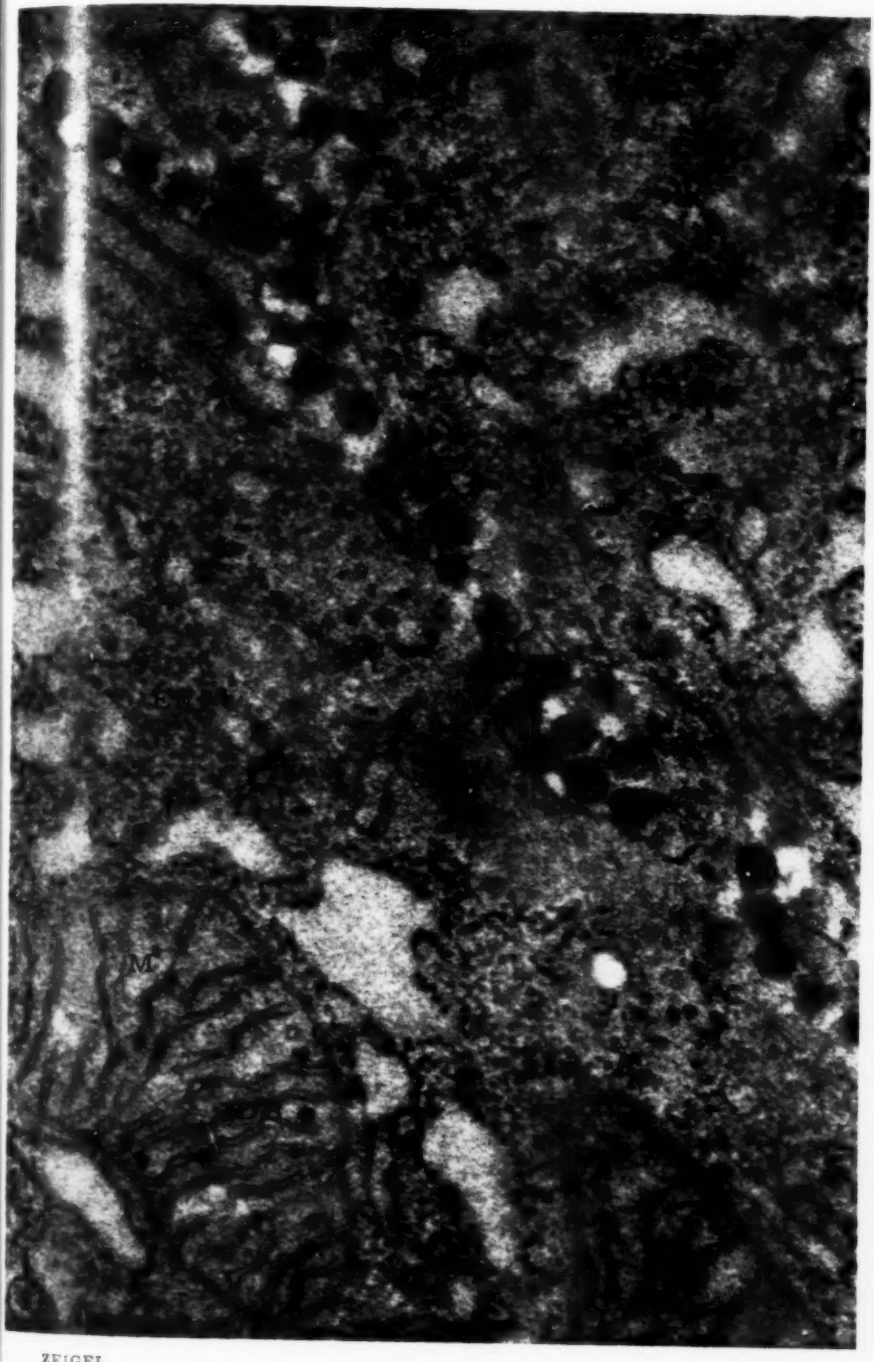
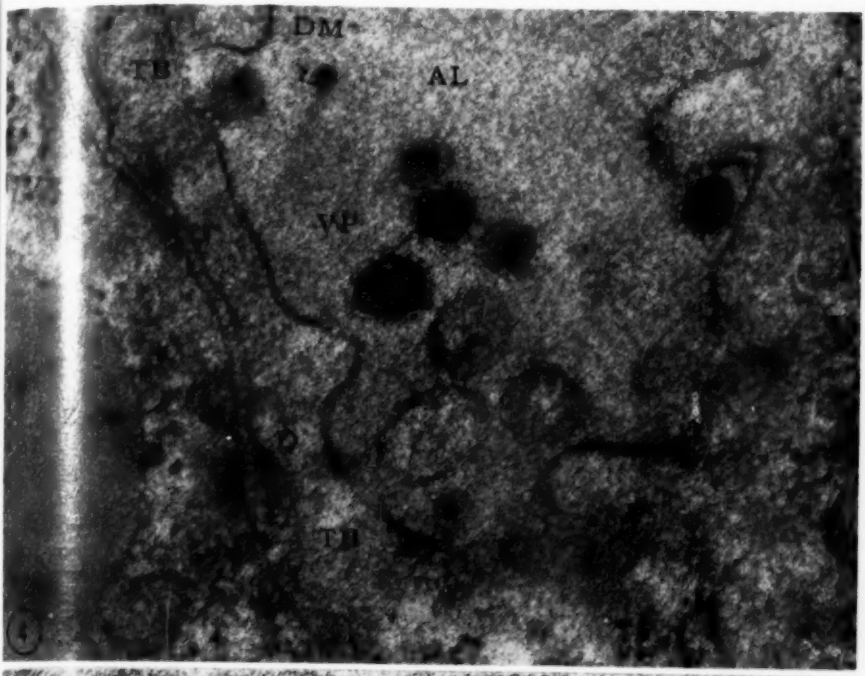

M

PLATE 140

FIGURE 4.—Several mature "virus particles" are observed in lumen of pancreatic acinus. Zymogen granules (Z) and terminal bar with specialized regions (desmosomes, D) are present. *Note* doubled character of apical plasma membrane (DM). Stained with lead acetate and ammonia vapors. Approximately × 120,000

FIGURE 5.—Region of pancreatic lumen in 8-day-old chick exhibits "virus particles" in various stages of maturation. *Note* in several particles the double nature of both inner and outer particle membranes. Outer dense ring (5th ring) is present in all particles when plane of section is equatorial. A doubled particle may also be observed (*arrow*), and it is suggested that these structures are due to two particles forming from the plasma membrane in close proximity as they appear to be doing in figure 18. Several of these particles in this micrograph served as the source of measurements for the diagrammatic representation in text-figure 1. Stained with lead subacetate (monobasic lead acetate). Approximately × 120,000


DM
TB
AL
VP
TB

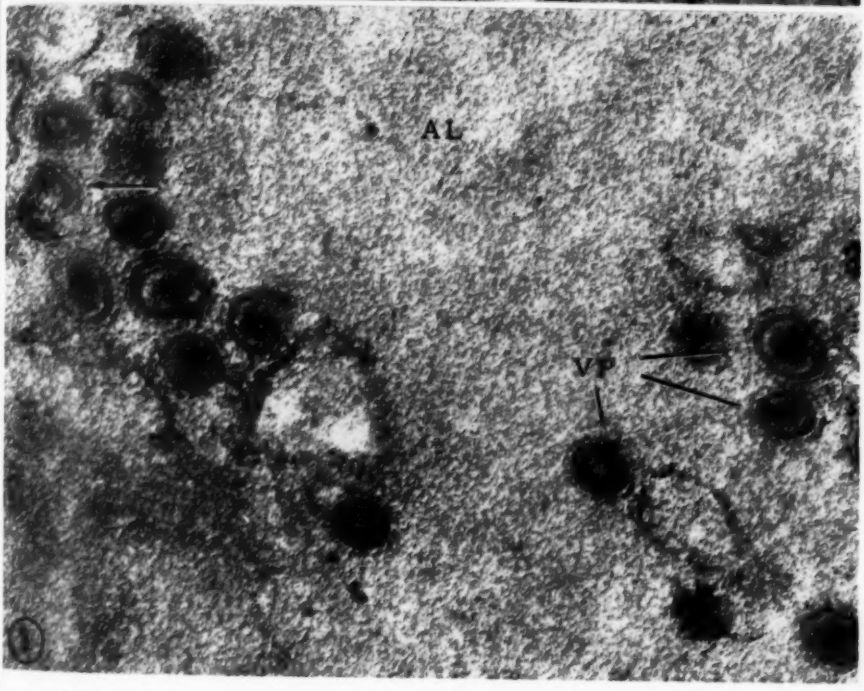
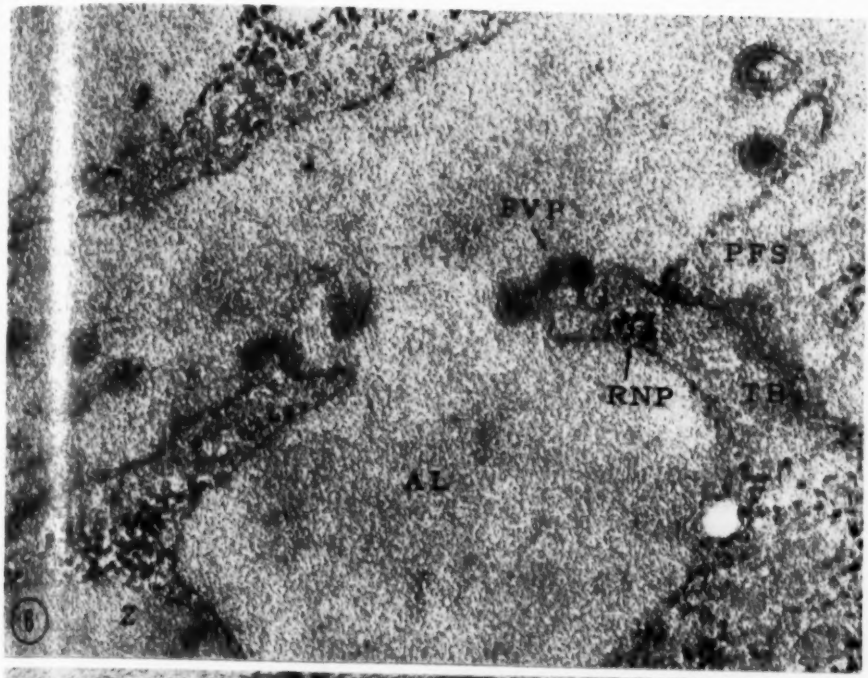

AL
VP

PLATE 141

FIGURE 6.—Portion of acinar lumen (AL) with associated zymogen granule (Z) may be seen. Terminal bar (TB), RNP particles, and RNP-particle-free space are present. Two "virus particles" are in the process of budding from apical plasma membrane. The double nature of their 2 membranes is evident as well as association with 5th outer ring, which does not appear to be of typical membranous nature. Small particles apparently composing this ring are not associated with apical plasma membrane where "virus particles" are not forming. Stained with lead subacetate. Approximately × 87,000

FIGURE 7.—Region of acinar lumen (AL) of 8-day-old chick contains "virus particles" in various stages of development at terminations of elongated processes. These processes are continuous with cytoplasm of acinar cells and bounded by apical plasma membrane. Double nature of inner and outer membranes of "virus particle" is evident as well as 5th outer dense ring (*arrow*). RNP-particle-free space is evident beneath apical plasma membrane in region where particles are in formative stages. Stained with lead subacetate. Approximately × 103,000


PVP
PFS
RNP
AL
B
Z

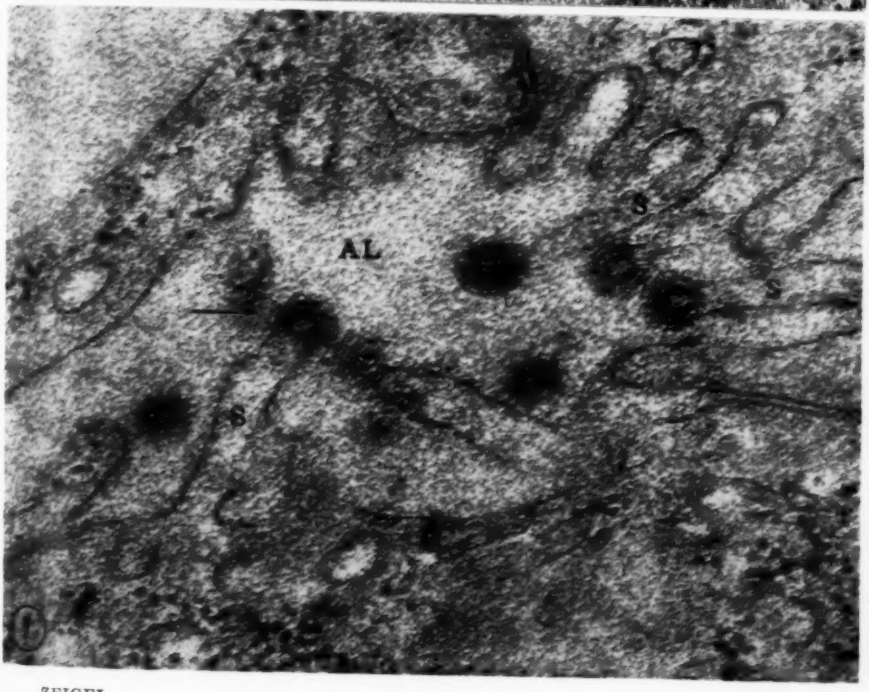
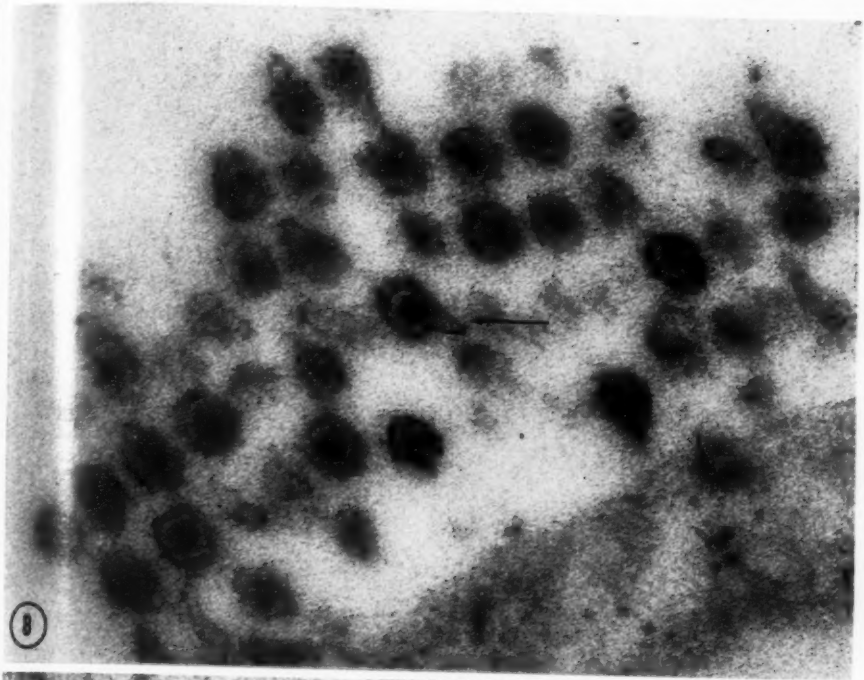

AL
S
S
S
C

PLATE 142

FIGURE 8.—Mature particles which are morphologically identical to virus particles described in various avian tissues are observed at base of acinar cell in pancreas of 7-day embryo. In several places the double nature of outer particle membrane may be observed. Frequently membranes appear to be distorted (*arrow*), which is apparently a characteristic of particles not in process of budding. No stain. Approximately × 125,000

FIGURE 9.—Several mature particles containing centrally located dense nucleoids are observed in lumen of acinus in 8-day-old chick. Cross sections of probable microvilli (MV) are indicated. In micrographs of good quality there is little danger in confusing these profiles with those of "viral particles." The acinar cells demonstrate terminal bars (TB) and abundant ergastoplasm (ER). Small, dense particles are observed to be associated with outside of "virus particles" 1, producing a cartwheel effect. "Virus particle" 2 is enveloped by distorted outer membrane. Stained with lead acetate and ammonia vapors. Approximately × 81,000



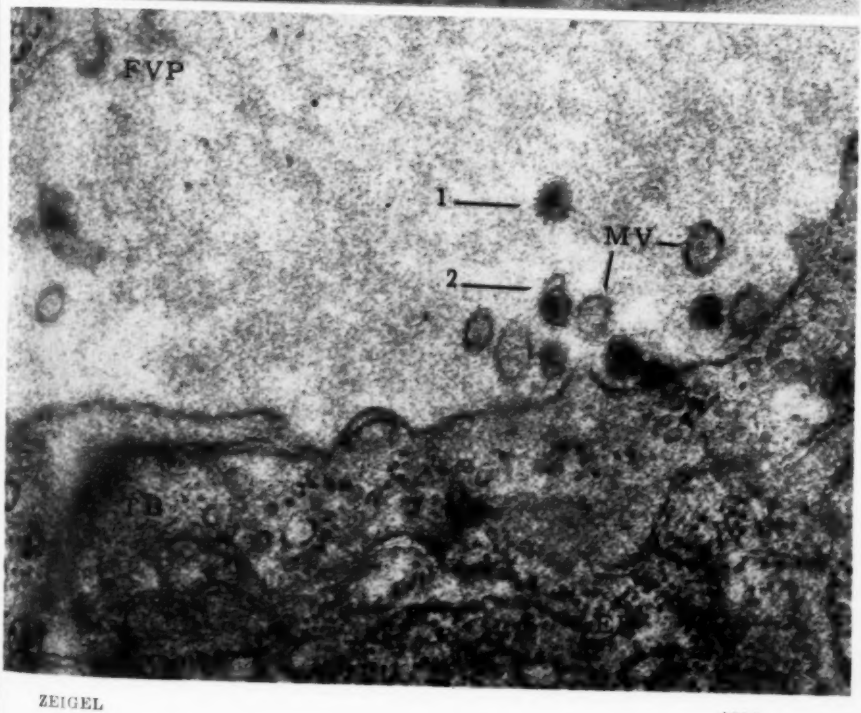

FVP
1
2
MV

PLATE 143

Figures 10 through 17 illustrate "virus particles" in various stages of "budding" from apical plasma membrane of pancreatic acinar cells in 8-day-old chick

FIGURE 10.—"Virus particle" in presumably an early stage of formation is demonstrated. Portion of terminal bar (TB), several RNP particles, and RNP-particle free space (PFS) are illustrated. No stain. Approximately × 120,000

FIGURE 11.—"Virus particle" in which inner membrane is beginning to thicken (*pointer*) is demonstrated "budding" from the apical plasma membrane. RNP-particle free space is observed to underlie region of particle formation. No stain. Approximately × 120,000

FIGURE 12.—Two "virus particles" in which nucleoids are beginning to form are illustrated. It is impossible to tell whether particles are still attached to cell via membrane connection out of plane of section. No stain. Approximately × 120,000

FIGURE 13.—Progressive stage in development of dense nucleoid in "virus particle" may be observed. *Note* double character of plasma membrane (DM). No stain. Approximately × 120,000

FIGURE 14.—Two "virus particles," 1 and 2, may be observed. Particle 1 is associated with stalk (S). Particle 2 appears to be enlarged; nucleoid region is not dense. No stain. Approximately × 120,000

FIGURE 15.—Two mature particles are observed. Particle 1 exhibits distorted membrane and particle 2 is associated with material which gives it cartwheel appearance. No stain. Approximately × 120,000

FIGURE 16.—Occasionally abnormally shaped "virus particles" are observed to bud from apical plasma membrane of acinar cells. This particle (*pointer*) possesses characteristic inner and outer membranes with associated 5th outer ring, though it is elongated and not typical. It is attached to cell by elongated process. No stain. Approximately × 120,000

FIGURE 17.—Oblique section through "virus particle" illustrates membrane of the associated stalk (S) and various concentric components of particle. Stained with Palay's modification of a lead-hydroxide technique. Approximately × 120,000

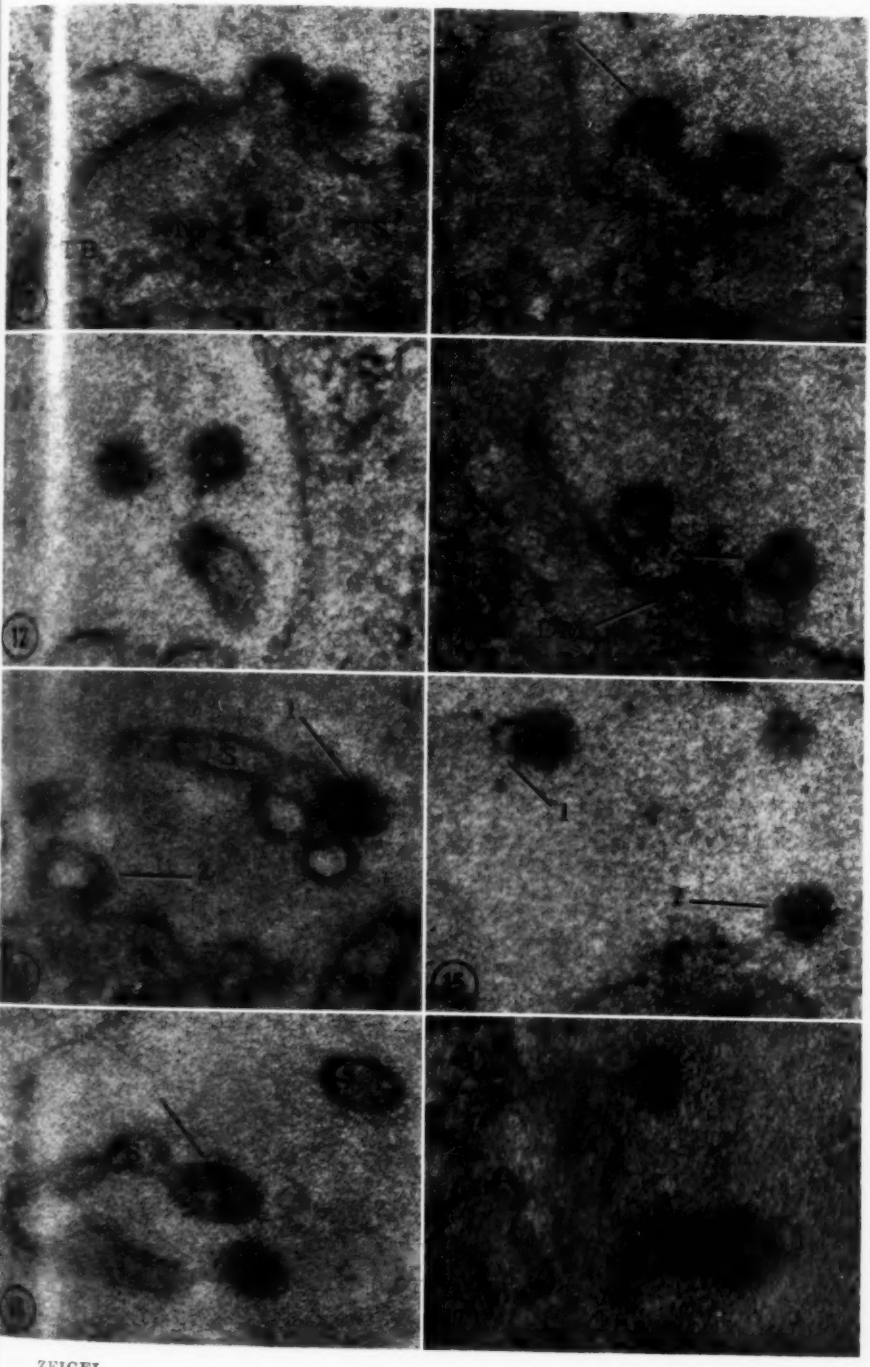


PLATE 144

FIGURE 18.—Acinar lumen of 3-day-old chick demonstrates several particles in process of formation. Two particles (*arrow*) may be seen forming in close proximity. It is possible that "double" particles such as in figure 5 are result of adjacent formation such as this. Stained with lead acetate and ammonia vapor. Approximately × 99,000

FIGURE 19.—Lumen of acinus in 3-day-old chick contains morphological entities, identical to virus particles described in other avian systems, in quantities suggestive of packed particles from pelletized material. Acinar lumen (AL) and zymogen granules (Z) are represented. Stained with lead acetate and ammonia vapors. Approximately × 48,000

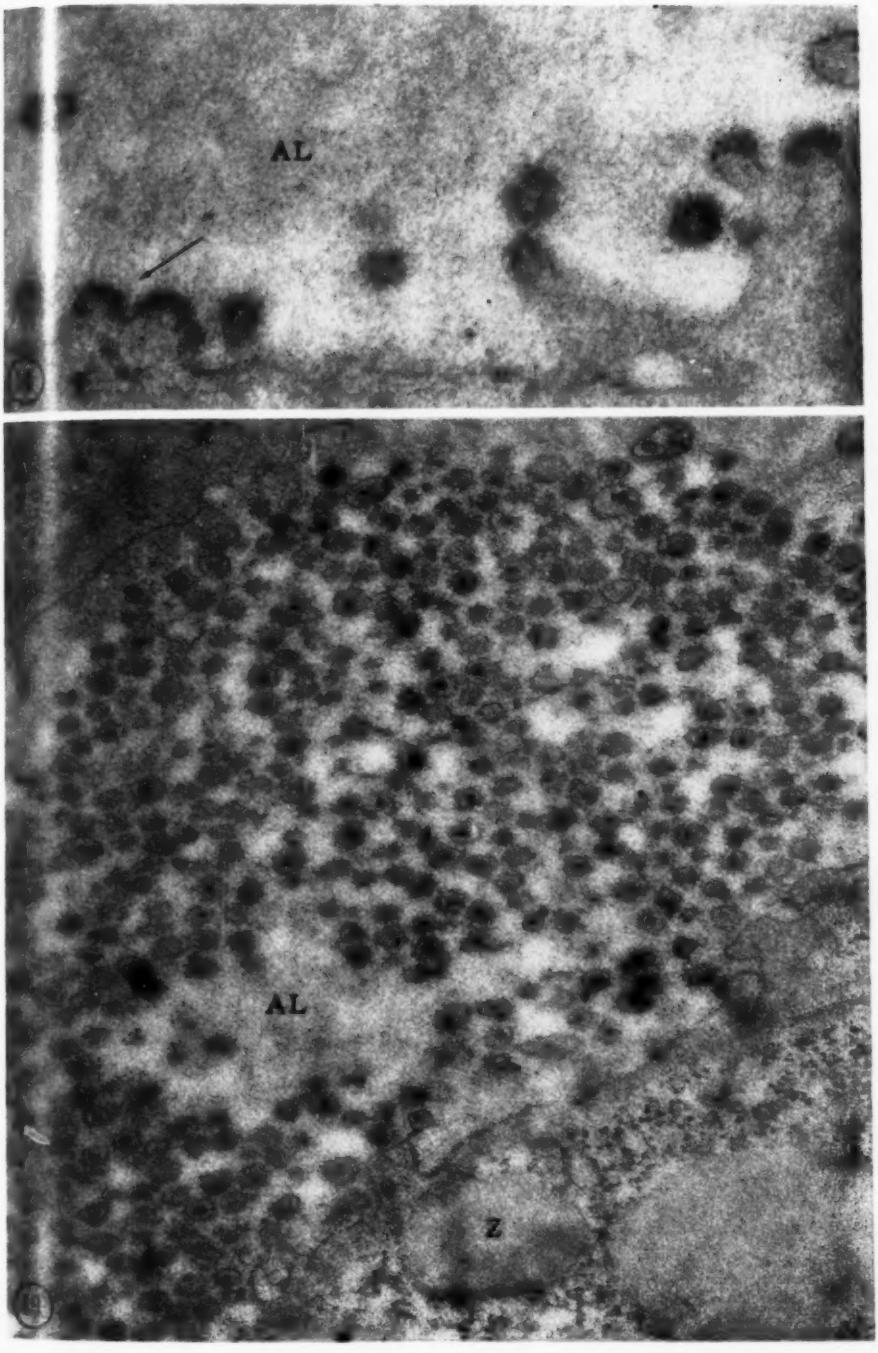

AL
AL
Z

PLATE 145

FIGURE 20.—This micrograph illustrates region near respiratory alveolar lumen (RAL) in 3-day-old chick whose pancreas was positive for "virus particle" formation. Epithelium (E) and associated basement membrane (BM) are indicated. Particles appearing identical to virus particles described in various other normal and neoplastic tissues in domestic fowl are present (*arrows*). They are observed in intercellular spaces in this particular micrograph, though they may occur in intracytoplasmic vesicles and vacuoles. Stained with lead acetate and ammonia vapors. Approximately × 44,000

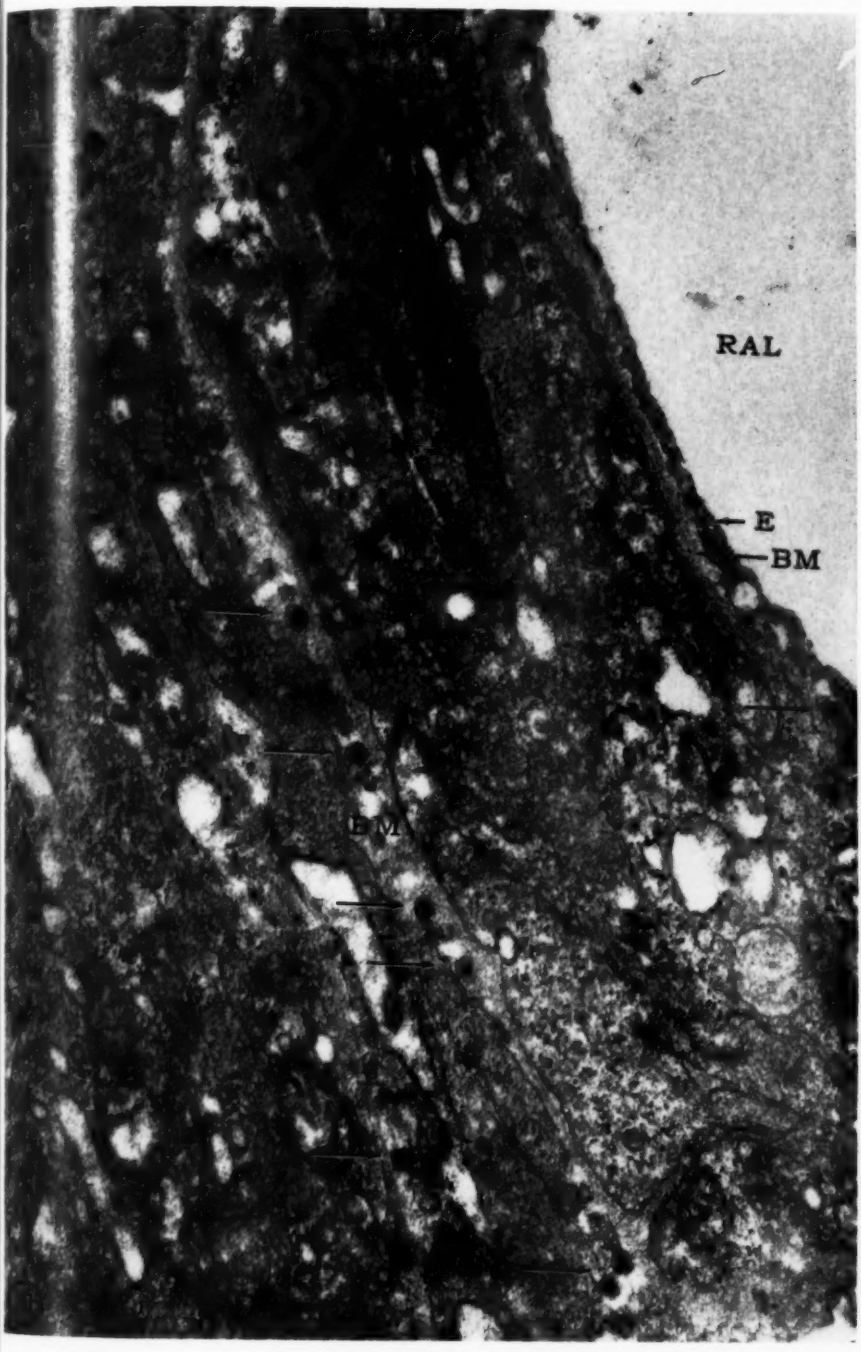

RAL
E
BM

PLATE 146

FIGURE 21.—Basal region of cells lining distal convoluted tubule in 3-day-old chick whose pancreas was positive for "virus particle" formation is illustrated. Portion of lumen of blood capillary (BC), endothelium (EN), basement membrane (BM), and several cell processes of distinctive character (CP) are presented. The mitochondria (M) are prominent and possess numerous cristae. "Virus particles" are designated by *arrows*. Stained with lead acetate and ammonia vapors. Approximately × 44,000

CP
CP
BM
BC

Announcements

Manuscripts Approved for JNCI, February 15 to March 15, 1961

"Host factors in cell culture. Further studies on the growth-controlling action of fresh human serum," by Erkki Saxén and Kari Penttinen.

"Synergism between small doses of total-body and local X radiation to transplanted mouse tumors," by Melvin R. Sikov and James E. Lofstrom.

"A spontaneous metastasizing fibrosarcoma in the golden hamster (*Mesocricetus auratus*) with some observations on tumor recurrence and metastasis after transplantation," by Michael Klein.

"The histology of sex-specific graft rejection," by E. J. Eichwald and E. C. Lustgraaf.

"Chromosomes of tumor cells. II. The effect of various liver carcinogens on mitosis of hepatic cells," by M. M. Maini and H. F. Stich.

"A and B antigens of tumors arising from urinary epithelium," by H. E. M. Kay and D. M. Wallace.

"Observations on inclusion bodies in normal and pathologic mucosa of the colon in man," by Benito Monis and Albert I. Mendeloff.

"Epithelial and stromal roles in carcinogenesis. II. Tumor development in internal transplants of skin and epithelium treated with 3-methylcholanthrene," by Charles A. Ashley.

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